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The Influence of a Male-Derived Protein Factor on Salivary Gland Degeneration in the Ixodid Tick, *Amblyomma hebraeum* Koch.

by

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Lee Owen Lomas

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of DOCTOR OF PHILOSOPHY.

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Edmonton, Alberta

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Figure 16.4, page 283; "Dorsal view of
Ornithodoros moubata
vagina".

Figure 17.1, page 306
in an unfed male
andersoni".

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Figure 1.2 General morphology of the adult ixodid tick reproductive tract. A. Male genital tract: T, testis; Vd, vas deferentia; SV, seminal vessicle; AG, accessory glands; GA, genital atria; ED, ejaculatory duct. B. Female genital tract; O, ovary; Od, oviduct; Cv, cervical vagina; SR, seminal receptacle; Vv, vestibular vagina; GA, genital aperture; TAG, tubular accessory gland; LAG, lobular accessory gland; LG, longitudinal groove. Modified after Sonenshine (1991) with permission from Oxford University Press.

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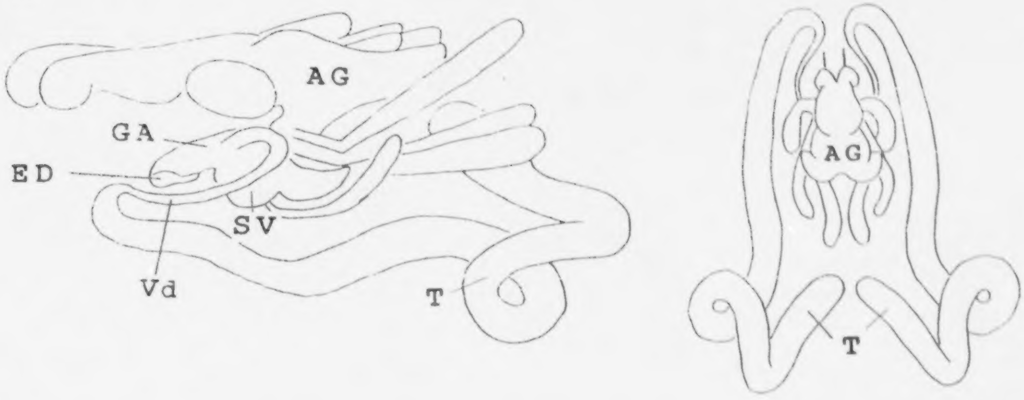
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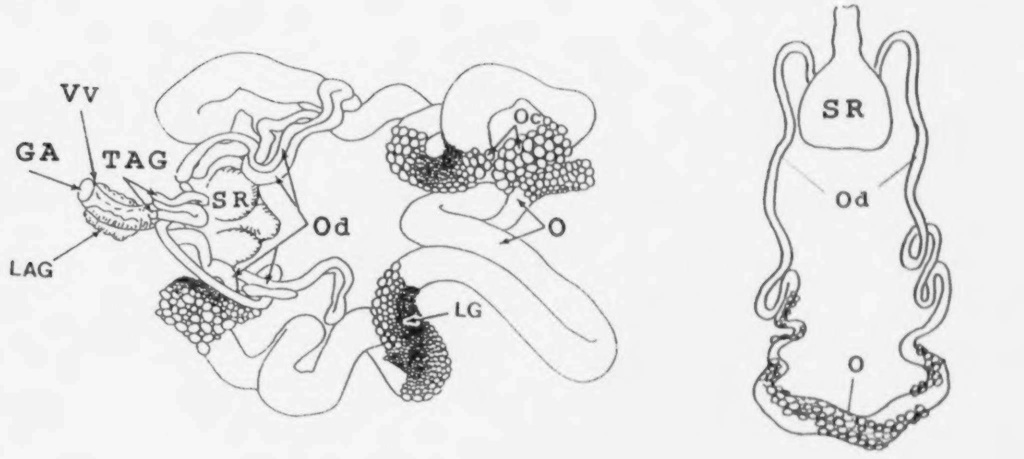
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16

roductive System

ucture, and physiology of the
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will be compared.
of intense interest for many
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th the Ixodidae and Argasidae,
ed. In the Ixodidae, this system
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(absent in argasid ticks), the
the **genital aperture** (Figs 16.1,
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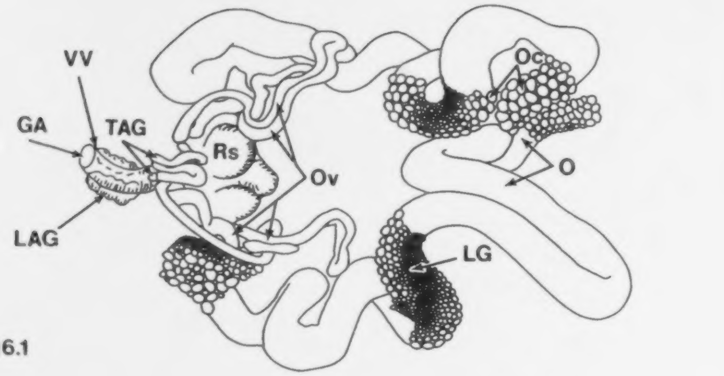

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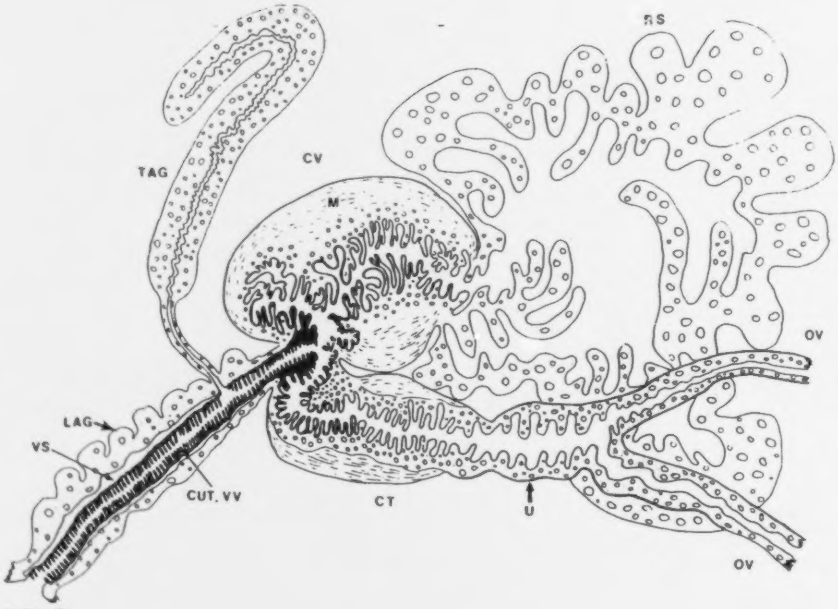

16.2

Figures 16.1, 16.2 Diagrams illustrating the reproductive system in female ixodid ticks. **16.1**. Dorsal view of the reproductive system of a mated, replete (72 hours after engorgement) female *Dermacentor andersoni*. Details of the genital aperture not shown. Modified after Brinton and Oliver (1971a), with permission from the *Journal of Parasitology*. **16.2.** Lateral view of the anterior region of the reproductive organs of a feeding virgin female (attached 7 days) *D. variabilis*. The ovary is omitted. After Sonenshine et al. (1985a), with permission from the *Journal of Chemical Ecology*. CV = Cervical vagina; Cut. VV = cuticular lining of the vestibular vagina; CT = connecting tube; GA = genital aperture; LAG = lobular accessory gland; LG = longitudinal groove; M = muscle; Oc = oocytes; O = ovary; Ov = oviduct; Rs = receptaculum seminis (seminal receptacle); TAG = tubular accessory gland; U = uterus; VV = vestibular vagina; VS = vestibular sinus.

lar accessory gland (LAG),
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ole is the glandular **Gené's**
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ing to a small sac connected
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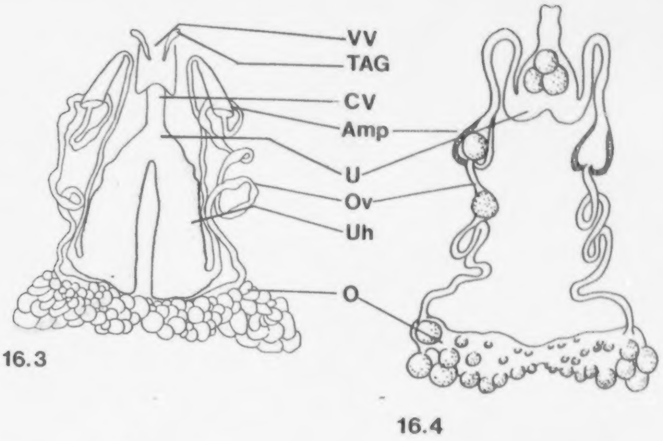
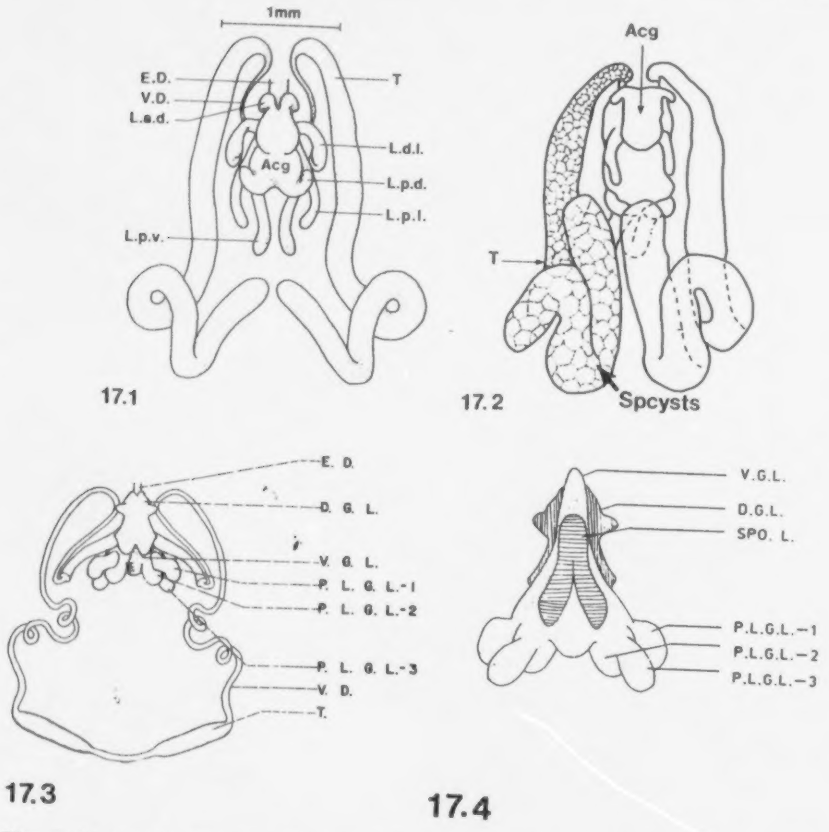


Figures 16.3, 16.4 Diagrams illustrating the reproductive organs of female argasid ticks. **16.3.** Dorsal view of a fed, mated female *Ornithodoros kelleyi*. The large uterus has a common anterior region and a large bifurcate posterior region (uterine horns). There is a prominent ampulla in the oviducts. After Sonenshine (1970b), with permission from the Entomological Society of America. **16.4.** Dorsal view of a fed, mated female *Ornithodoros moubata*. Ova are shown migrating through the oviducts and accumulating in the uterus. The cervical vagina and the tubular accessory glands are omitted. After Aeschlimann (1958), with permission from *Acta Tropica*, Elsevier Science Publishers, Amsterdam. Amp = Ampulla; CV = cervical vagina; O = ovary; Ov = oviducts; TAG = tubular accessory glands; U = uterus; Uh = uterine horns (bifurcate region of the uterus); VV = vestibular vagina.

undeveloped oogonia and primary oocytes project from its wall. Histologically, the ovary is similar in both ixodid and argasid ticks (Figs 16.5–16.7). In the unfed female *D. andersoni*, the ovary is approximately 6 mm long and 64–110 μm in diameter. In ixodid ticks, a longitudinal groove occurs along one side of the ovary (Figs 16.1, 16.5, 16.8). Germinal cells in the longitudinal groove are generally less developed than elsewhere in this organ, i.e., this appears to be a region of slower developmental activity. Symbiotic rickettsia-like intracellular microbes (*Wohlbachia* sp.) (Rickettsiales) occur in the interstitial cells and in the oocytes. The oocytes in the unfed female ovary, according to the classification system of Balashov (1972), are stage 1 oocytes (see below, Oogenesis). The oocytes and oogonia are predominantly round in shape. They communicate with one another by intercellular bridges (Brinton and Oliver, 1971b). The cytoplasm is dense, and filled with numerous free ribosomes. In contrast to the insects, the tick ovary is not segmented into separate zones, such as the germarium or vitellarium, each with differing rates of reproductive development as occurs in the different ovarioles of the insect ovary. Figure 16.8 summarizes the changes in the ovarian wall and the different stages of oogenesis.

During feeding of ixodid ticks (or, in argasid ticks, after feeding), the oocytes enlarge and transform into more advanced stages, migrating to a position



Figures 17.1–17.4 Diagrams illustrating the structure of the male reproductive system in ixodid and argasid ticks. **17.1**. Structure of the reproductive system in an unfed male ixodid tick, based on *Dermacentor andersoni* **17.2.** Structure of the reproductive system in a fed male ixodid tick. The long, coiled testis is filled for most of its length with numerous spermatocysts (small, irregularly shaped clusters outlined by dotted lines), each containing spermatogonia, primary spermatocytes, secondary spermatocytes or spermatids. The vasa deferentia and the ejaculatory duct are obscured by the massive male accessory gland and are not shown in the figure. **17.3.** Structure of the reproductive system in an unfed argasid tick (*Ornithodoros kelleyi*), dorsal aspect. **17.4.** Structure of the accessory gland in an unfed argasid tick (*O. kelleyi*), ventral aspect. Acg = Accessory gland; D.G.L. = dorsal granular lobe; E.D. = ejaculatory duct; L.a.d. = antero-dorsal lobe; L.d.l. = dorso-lateral lobe; L.p.d. = postero-dorsal lobe; L.p.l. = postero-lateral lobe; L.p.v. = postero-ventral lobe; P.L.G.L. 1–3 = postero-granular lobes 1–3; Spcysts = spermatocysts; Spo. L. = spongy lobe; V.D. = vas deferens; V.G.L. = ventral granular lobe; T = testis. Figure 17.1 after Douglas (1943), with permission from the University of California Press; Fig. 17.2 from Oliver and Brinton (1972), with permission from the *Journal of Parasitology*; Figs 17.3–17.4 from Sonenshine (1970b), with permission from the Entomological Society of America.

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Department of Zoology

Lee O Lomas

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Figure 1.3 Position and general morphology of the salivary glands. SG, salivary gland; O, ovary; SR, seminal receptacle; MT, malphigian tubules. i) Type I acini: CC, central cell; PC, pyramidal cell; NC, neck cell; SD, salivary duct. ii) Type II acini: AbIC, abluminal interstitial cell; AdIC, adluminal interstitial cell; A, a-cell; B, b-cell; C, c-cell; Vl, valve; L, lumen. iii) Type III acini: D, d-cell; E, e-cell, F, f-cell. Modified from Megaw and Beadle (1979).

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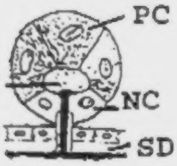

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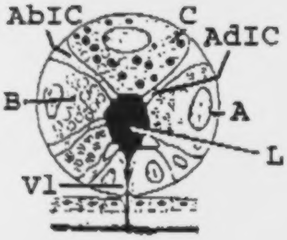
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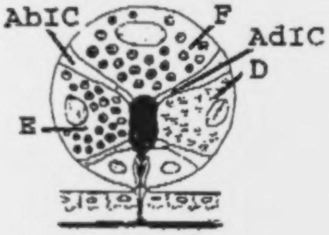
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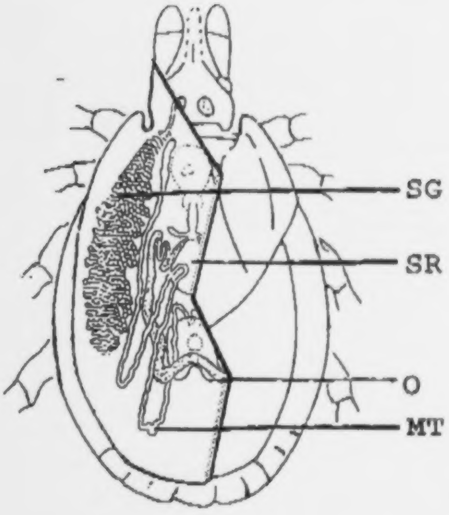

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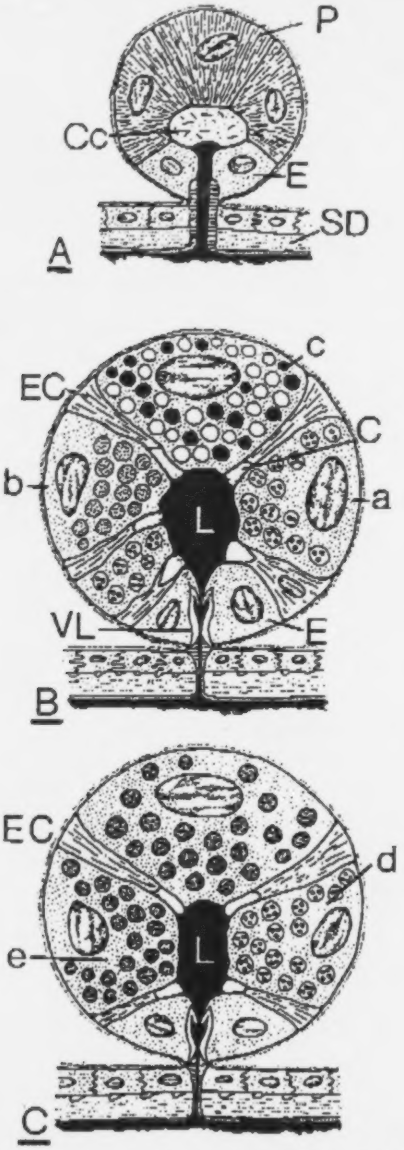

Fig. 1. (A): Type I acinus: pyramidal cells (P) are separated from lumen by a central cell (Cc). Epithelial cells (E) attach acinus to duct (SD). Note absence of a valve guarding acinus lumen; (B): Type II acinus: these acini contain three types of granule-secreting cells, 'a', 'b' and 'c'. Epithelial cells (EC) running between granule-secreting cells are cut off from lumen (L) by cap cells (C). Lumen of acinus is separated from duct system by a cuticle-lined valve (VL); (C): Type III acinus: although similar to type II acini, these acini contain only two types of granule-secreting cells 'd' and 'e'.

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>
> Figure 12, page 716; "Semidiagrammatic drawing of the highly convoluted ovary (Ov), oviducts (Od), seminal receptacle (Sr)(distended by the presence of endospermatophores), and accessory gland lobes (Ag), as observed *in situ*, 72 hr after completing engorgement. Longitudinal groove (Lg), oocyte (Oc)".

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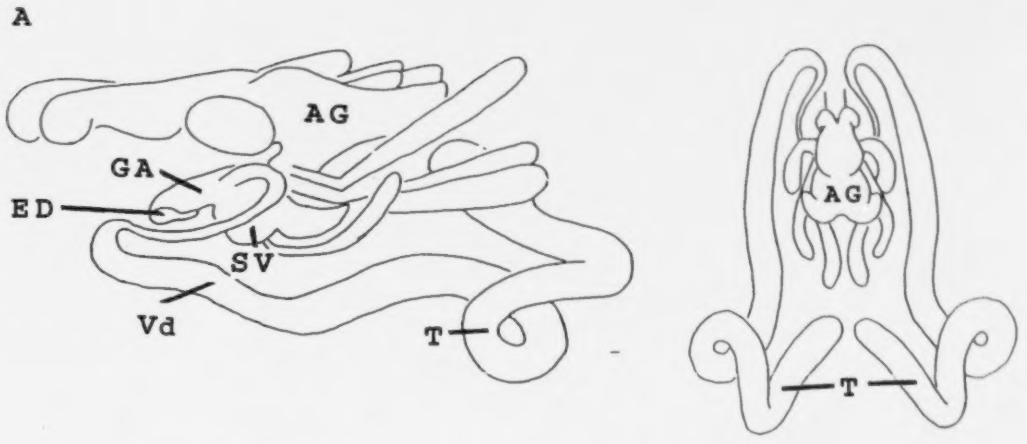
Sincerely,

Lee O Lomas.

Lee O Lomas

Figure 1.2 General morphology of adult ixodid tick reproductive structures. (A). Male genital tract: T, testis; Vd, vas deferentia; SV, seminal vesicle; AG, accessory glands; GA, genital atria; ED, ejaculatory duct. Modified from Douglas (1943) and Sonenshine (1992). (B). Female genital tract; O, ovary; Od, oviduct; SR, seminal receptacle; Vv, vestibular vagina; GA, genital aperture; TAG, tubular accessory glands. Modified from Brinton and Oliver (1971) and Sonenshine (1992).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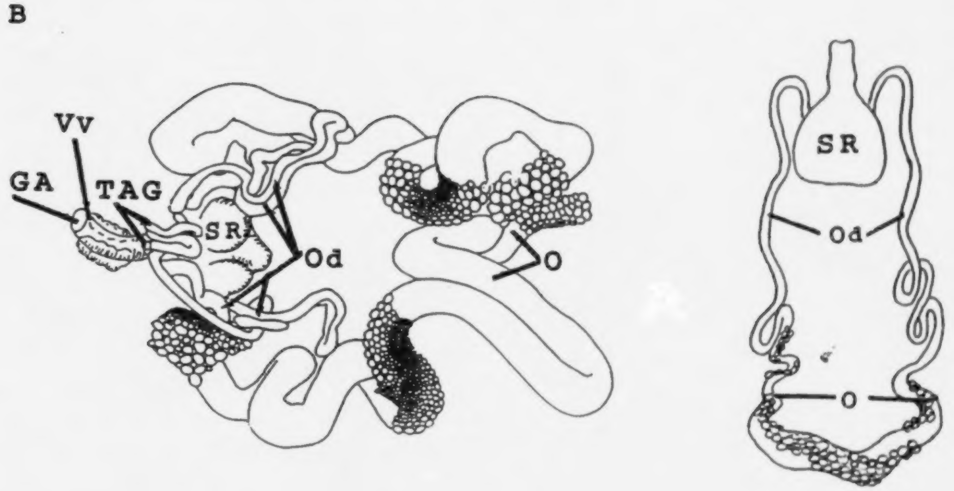
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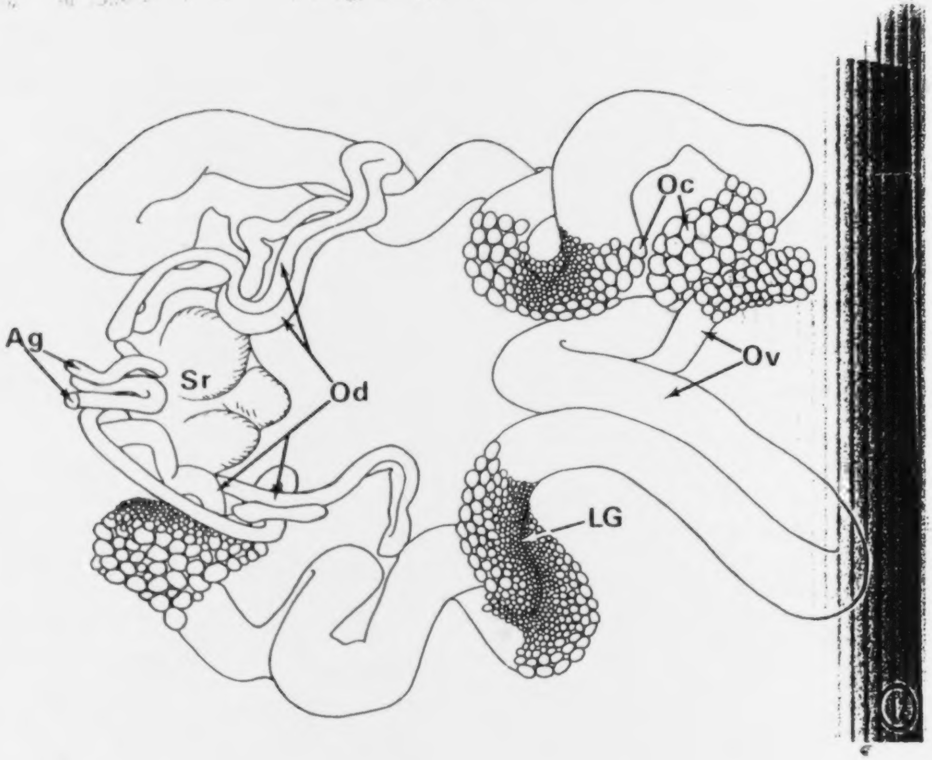
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Figure 12 (page 716)

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Date: August 12, 1993

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FACULTY OF GRADUATE STUDIES AND RESEARCH

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled: The Influence of a Male Derived Protein Factor on Salivary Gland Degeneration in the Ixodid Tick, *Amblyomma hebraeum* Koch, submitted by Lee Owen Lomas in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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Dedicated to my Grandmothers

Elizabeth Louisa Lomas

and

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Abstract:

The salivary glands are the primary osmoregulatory organs of feeding ixodid ticks and are capable of secreting copious amount of fluid into the host. Once engorged, the tick drops from the host and the salivary glands degenerate within 4 days. Degeneration is initiated by the release of an ecdysteroid and controlled by at least two events; feeding beyond a critical weight (300-400 mg in Amblyomma hebraeum) and mating. If females remain virgin, most do not feed beyond the critical weight. Of the few virgins that do reach a size above the critical weight, salivary gland degeneration is delayed by 3-4 days. This delay, however, could be prevented by injecting a homogenate of male gonad into the haemocoel of virgin females.

The male genital tract contains a proteinaceous 'male factor' of apparent molecular weight 20-60 kD and density 1.18-1.21 g/ml. Male factor is not a 'prostaglandin E_2 like factor', as injection of PGE_2 increases the viability of salivary glands in both mated and virgin females above the critical weight. This male factor resides primarily within the testis-vas deferens-ejaculatory duct and is distinct from the spermatozoa. No male factor activity was found in the accessory-gland portion of the genital tract. Male factor like activity is also found in the ovary of mated females and the synganglion of both fed males and females, and the haemolymph of mated females, but not in salivary gland homogenates of fed males.

As male factor activity is easily detected in the haemolymph, it likely gains access to its target tissue as a humoral factor. Although the specific target remains unknown, it is unlikely to be the salivary glands themselves. Male factor acts to stimulate the synthesis/release of ecdysteroids in the haemolymph. Injection of male factor into virgin females, which normally have a low haemolymph ecdysteroid titre, causes a significant rise in the

haemolymph ecdysteroid titre to a level similar to that of mated females. The endocrine source of ecdysteroids is still unknown; various culturing methods have so far failed to demonstrate synthesis of ecdysteroids *in vitro*.

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Chapter 1
Introduction

Ticks:

Ticks are a group of highly successful blood feeding arthropods that parasitize birds, mammals, and reptiles. Their dependence on host blood combined with a prolonged feeding period also makes them particularly well-suited for transmitting pathogenic organisms to their host. Indeed, ticks are second only to mosquitoes in terms of the impact on humans and their domestic stock. Recently, there has been a resurgence of new (or previously unknown) tick borne diseases, such as Lyme disease (causative agent: *Borrelia burgdorferi*; vector: *Ixodes* sp.; Burgdorfer *et al.*, 1982) and human ehrlichiosis (causative agent: *Ehrlichia chaffeensis*; vector: *Amblyomma americanum*; Olson, 1992). In addition to transmitting pathogenic agents, ticks themselves cause considerable discomfort and damage to their hosts as a result of (1) anemia (death can result from severe tick infestations), (2) secondary infection at the feeding site (*e.g.*, Dermatophilosis caused by *Dermatophilus congolensis*; Norval, 1986), (3) an increased potential for general systemic infections due to a suppression of the host's immune system (Wikel and Ramchandra, 1992), and (4) toxicosis and/or tick paralysis due to the secretion of a toxin in the saliva (Gregson, 1973; Stone and Binnington, 1986). Overall, ticks are responsible for global economic losses in the billions of dollars annually (Sonenshine, 1991). Because of the medical and economic impact that ticks have, a considerable amount of research has been devoted to many aspects of tick ecology and physiology in an attempt to devise novel methods of control. The following review is intended to introduce aspects of tick biology that are relevant to this thesis.

Tick Phylogeny:

Ticks are believed to have initially evolved as obligate parasites of reptiles during the mid-late Paleozoic (Savory, 1977; Hoogstraal, 1983; Hoogstraal and Kim, 1985). As reptiles went through a period of adaptive radiation during the early Mesozoic, ticks concurrently evolved into three evolutionary lines, presently represented by the families Ixodidae, Nuttalliellidae, and Argasidae. During the late Mesozoic, when birds and mammals replaced reptiles as the dominant life forms, ticks went through a second period of adaptive radiation, resulting in the division of Ixodidae into two phyletic lines, the prostriata and the metastriata. Although a few tick genera retain a predilection for reptilian hosts, most tick species are now parasites of birds or mammals (Hoogstraal, 1983). Today, there are about 840 tick species divided predominantly among the two families Ixodidae (670 spp.) and Argasidae (170 spp.; figure 1.1). The family Nuttalliellidae contains only one species, *Nuttalliella namaqua*.

Ixodid Tick Life Cycle:

The life cycle of ixodid ticks consists of 4 stages: egg, larva, nymph, and adult. Each instar must take a blood meal in order to proceed to the next developmental stage. This blood meal lasts several days to several weeks, depending on the species (reviewed by Balashov, 1972). Except in adult males, the feeding period is divided into three phases. The first is a preparatory phase, lasting 12-24 hrs, where the tick secretes a variety of substances into the host to establish and maintain the feeding lesion. These substances include a cement that holds the mouth parts in the feeding lesion (Moorhouse, 1969), anticoagulants and vasodilators that increase blood flow to the lesion (Ribeiro, 1987), and immunosuppressive substances that suppresse the host immune response (Ribeiro, 1987; Wikel and Ramchandra,

Figure 1.1 Phylogenetic relationship of ticks. Dendrogram modified from Hoogstraal (1983) and Sonenshine (1991).

Phylum Arthropoda
Subphylum Chelicerata
Class Arachnida
Subclass Acari
Order Acarina
Suborder Ixodida
Superfamily Ixodoidea

- Ixodidae
  - Prostriata
    - Ixodinae
      - Ixodes 245 spp.
  - Metastriata
    - Amblyomminae
      - Amblyomma 102 spp
      - Aponomma 24 spp.
    - Haemaphysalinae
      - Haemaphysalis 155 spp
    - Hyalomminae
      - Hyalomma 30 spp.
    - Rhipicephalinae
      - Margaropus 3 spp.
      - Boophilus 5 spp.
      - Rhipicephalus 70 spp.
      - Nosomma 1 spp.
      - Cosmiomma 1 spp.
      - Dermacentor 30 spp.
      - Rhipicentor 2 spp.
      - Anomalohimalaya 3 spp
- Nuttalliellidae
  - Nuttalliellinae
    - Nuttalliella 1 spp.
- Argasidae
  - Argasinae
    - Argas 56 spp.
  - Ornithodorinae
    - Ornithodoros 100 spp.
  - Otobinae
    - Otobius 2 spp.
  - Nothoaspinae
    - Nothoaspis 1 spp.
  - Antricolinae
    - Antricola 8 spp.

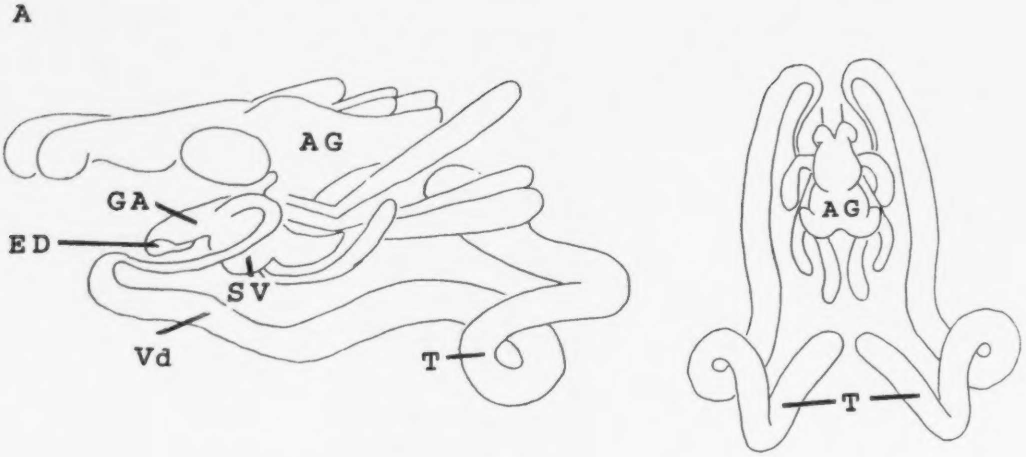
1992). The second phase, and longest in duration, is a slow feeding phase where the tick alternates between periods of blood intake, salivation, and rest. During this time, a considerable amount of host blood is taken, and the nutrient portion is concentrated in the gut (Lees, 1946) by the elimination of excess fluid into the host via the salivary glands (Tatchell, 1967; Kaufman and Phillips, 1973; Kaufman, 1983). Ixodid ticks increase their body weight about 10-fold during this phase. The third phase, known as the rapid expansion phase, occupies the final 12-24 hrs of the normal feeding cycle. During this phase, the tick imbibes the largest volume of blood, increasing her body weight an additional 10-fold, then detaches from the host. In the case of the immature stages, a moult will occur within 30-50 days (reviewed by Balashov, 1972). In the adult female, a batch of several thousand eggs is laid over several weeks, after which the female dies. The adult female enters the rapid expansion phase only if she has mated (Gregson, 1944; Pappas and Oliver, 1971, 1972). Virgin females do not enter this final phase, and instead, feed slowly for an extended period of time (Gregson, 1944; Balashov, 1972; Oliver, 1989).

Reproductive Biology:

The Adult Female Genital System:

The genital system of newly moulted females consists of a U-shaped tubular ovary that opens distally into a pair of coiled or folded oviducts (figure 1.2). The oviducts continue forward, fusing into a single common oviduct that leads into the cervical vagina. Fused dorsally with the cervical vagina is the seminal receptacle, a repository for the sperm and male fluids transferred during mating. The cervical vagina continues anteriorly into a vestibular vagina, that ends at the genital aperture. Near the junction between the cervical and vestibular vaginas, a small pair of tubular accessory glands fuse with the vestibular vagina (reviewed by Khalil, 1970; Sonenshine, 1991).

Figure 1.2 General morphology of adult ixodid tick reproductive structures. (A). Male genital tract: T, testis; Vd, vas deferentia; SV, seminal vesicle; AG, accessory glands; GA, genital atria; ED, ejaculatory duct. (B). Female genital tract; O, ovary; Od, oviduct; SR, seminal receptacle; Vv, vestibular vagina; GA, genital aperture; TAG, tubular accessory glands. Figure 1.2(A) modified from: Douglas, J. R. 1943. Internal Anatomy of *Dermacentor andersoni* Stiles. University Publications in Entomology 7:207-282 with permission from University of California Press; and Sonenshine, D. E. 1991. Biology of Ticks, Volume 1. Oxford University Press, with permission from Oxford University Press Inc. Figure 1.2(B) modified from: Brinton, L. P. and Oliver, J. H. Jr. 1971. Gross anatomical, histological, and cytological aspects of ovarian development in *Dermacentor andersoni* Stiles. Journal of Parasitology 57:708-710 with permission from the American Society of Parasitologists; and Sonenshine, D. E. 1991. Biology of Ticks, Volume 1. Oxford University Press, with permission from Oxford University Press Inc.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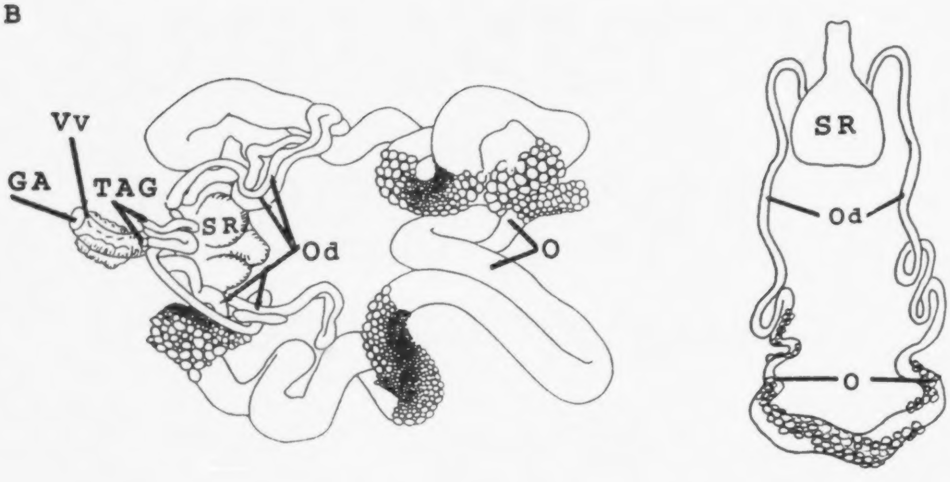
Lateral Dorsal


B
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TAG
SR
Od
O
SR
Od
O

Ventral Dorsal

Before feeding, oogonia and primary oocytes in less advanced stages of development occupy the inner wall of the ovary. Development of the primary oocytes does not occur beyond meiotic interphase until the female begins to feed (Khalil, 1970; Raikhel, 1983). During the slow feeding phase, the ovary increases greatly in size and the primary oocytes complete meiosis and enter a cytoplasmic growth phase (Khalil, 1970; Raikhel, 1983). Within 7-10 days after attachment, the female has usually mated, engorged and dropped from the host. The endospermatophore, containing the spermatids and seminal fluid and stored in the seminal receptacle, is dissolved and the sperm released into the connecting tube and common oviduct within 24 h of detachment (Khalil, 1970). Fertilization occurs shortly after host detachment, at which time the oocytes enlarge tremendously and vitellogenesis is initiated. Ovulation begins 5-7 days later, followed by oviposition.

In feeding virgin females, ovary development proceeds at a slower rate; even after 21 days of attachment, the ovary size does not exceed that of mated females attached for only 5 days (Khalil, 1970). Any oocytes that have started the cytoplasmic growth phase are resorbed within 24 days (Khalil, 1970).

The Adult Male Genital System:

Following ecdysis of the adult male, the genital system consists of a pair of elongate, tubular testes that connect anteriorly to paired coiled vas deferentia (figure 1.2). The vas deferentia widen distally, then fuse into a common seminal vesicle. A large multilobed accessory gland is also present, consisting of a combination of granular lobes and spongy lobes (El-Shoura, 1986). The common seminal vesicle and accessory glands empty into a common genital atrium that is continuous with the ejaculatory duct and end at the genital pore (Khalil, 1970).

Sperm development occurs in two stages; spermatogenesis (meiotic and mitotic divisions yielding haploid spermatids) and spermiogenesis (the growth and differentiation that produces reproductively viable sperm). Spermiogenesis is further divided into a growth and elongation phase (producing prospermia) and a capacitation phase (sperm maturation; Oliver and Brinton, 1972; Shepherd *et al.*, 1982). Following ecdysis, the testis contains clusters of primary spermatocytes. These clusters, known as spermatocysts, are imbedded in the testicular wall and surrounded by a thin epithelial layer (Raikhel, 1983). In almost all metastriate ticks (exceptions include *Amblyomma triguttatum*; Guglielmone and Moorhouse, 1983, and possibly *Aponomma hydrosauri*, *A. concolor*, and *A. varanensis*; Oliver, 1989), spermatogenesis is halted at the meiotic prophase until feeding begins (Khalil, 1970). Within 3 days of male attachment, the testes enlarge and meiotic division of the spermatocytes is reinitiated (Khalil, 1970). Within 5 days of feeding, spermiogenesis has begun and mature prospermia can be found throughout the testis. Capacitation, the final phase of sperm development, occurs only after the sperm have been transferred to the female during copulation. The process of capacitation requires 24 h and is initiated by a protein in the male accessory gland (Shepherd *et al.*, 1982; see below). This process produces mature, elongate spermatozoa, double the length of the original spermatid, that are capable of fertilization (Shepherd *et al.*, 1982).

Mating of Ixodid Ticks:

Mating of most metastriate ticks takes place on the host, is coordinated by pheromones, and is intimately associated with feeding. As mentioned above, a blood meal is required by both sexes prior to mating to allow for gamete and genital tract maturation. Feeding is also required for the production of pheromones, allowing the sexes to identify sexually responsive conspecific mates. Once females have attached and fed for a few days, they release three classes of sex pheromones: attractant sex pheromones (*e.g.*. 2,6-dichlorophenol; Berger, 1972) that attracts feeding or fed males; a contact sex pheromone, composed of cholesterol esters localized on the cuticle, that allows the identification of receptive from non-receptive females (Hamilton and Sonenshine, 1988); and genital sex pheromones, a mixture of fatty acids and their methyl esters produced by the anterior portion of the reproductive tract, that allows for conspecific recognition (Allan *et al.*, 1988; Allan *et al.*, 1991). In addition, fed males from some *Amblyomma* genera produce an aggregation-attachment pheromone (Rechav *et al.*, 1976; Rechav *et al.*, 1977) that attracts both unfed males and females (Sonenshine, 1985). Although interspecific mating does occur (Oliver and Brinton, 1972), it is rare.

Once a suitable mate is found, the male deposits a spermatophore onto the ventrum of the female. This spermatophore consists of a three layered external shell (the ectospermatophore), which encloses the sperm, seminal fluid, protein granules, and the endospermatophore (Feldman-Muhsam and Borut, 1971; Oliver and Brinton, 1972; Feldman-Muhsam *et al.*, 1973; Feldman-Muhsam, 1986). After the ectospermatophore is placed on the ventral surface of the female, the endospermatophore evaginates into the female's genital tract and the sperm mass is pushed into the everted endospermatophore. This process occurs without any intervention by the male. Once the endospermatophore transfer is complete, the ticks separate and the ectospermatophore is discarded. Although females may copulate several times before engorgement (Feldman-Muhsam, 1986), it is not known if they do so with the same or different males.

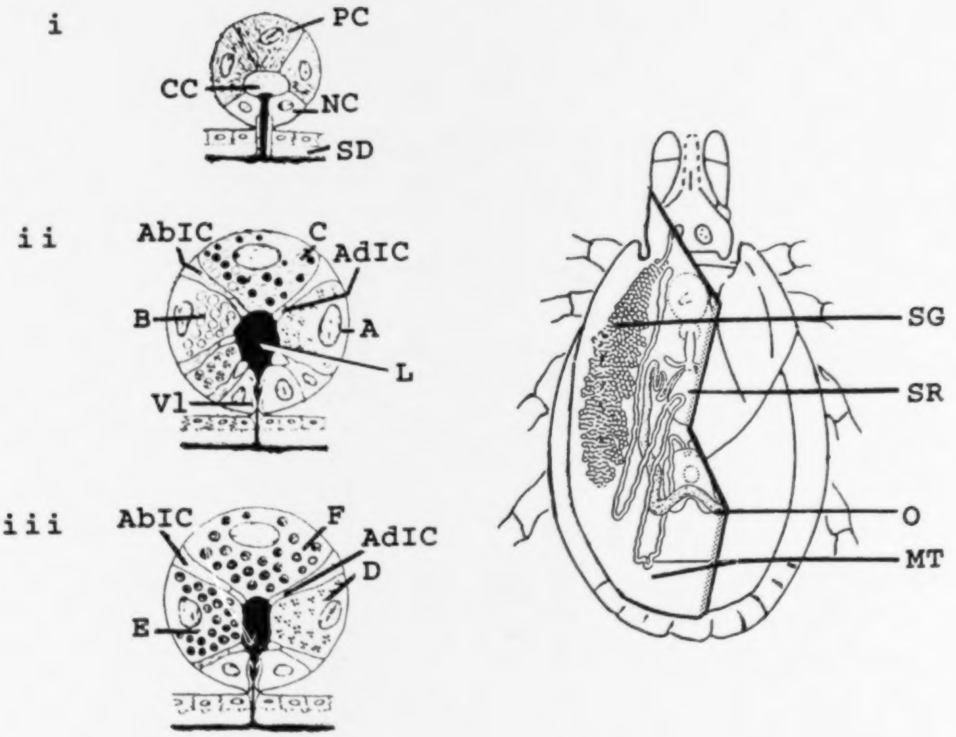
Tick Salivary Glands:

As previously mentioned, ixodid ticks feed to repletion over a period of 7-10 days, increasing their body weight by as much as 100-fold (Balashov, 1972). During this feeding period, the nutrient portion of the blood meal is concentrated in the gut (Lees, 1946) and the excess fluid secreted back into the host via the salivary glands (Tatchell, 1967; Kaufman and Phillips, 1973; Kaufman, 1983). Measurements from *Ixodes ricinus* (Lees, 1946), *B. microplus* (Tatchell, 1967), *Dermacentor andersoni* (Kaufman and Phillips, 1973), and *Amblyomma americanum* (Hsu and Sauer, 1975) suggest that from 30-70% of the ingested fluid can be secreted back into the host by the salivary glands. In a detailed study by Kaufman and Phillips (1973), *D. andersoni* females, which typically weigh 10 mg unfed and 700-800 mg engorged, were able to process as much as 4000 mg of host blood.

The salivary glands are two prominent paired organs situated laterally in the body cavity, extending from the anterior of the tick to the single pair of spiracles located posterior to the 4th pair of walking legs (figure 1.3). They are comprised of three acinar types in females and 4 in males, interconnected by a system of ducts that anastomose into a main duct and drains into the buccal cavity. Type I or 'agranular' acini are confined to the anterior region of the gland along the main duct and proximal portions of secondary ducts. Type II and type III acini contain both agranular and granular cell-types and are located primarily throughout the anterior two-thirds of the gland along secondary and tertiary duct branches. Probably the type III acini are primarily responsible for fluid elimination (reviewed by Kaufman, 1983).

Salivation is controlled by catecholaminergic nerves (Sauer *et al.*, 1979; Kaufman, 1983). Both dopamine and norepinephrine have been identified in salivary gland homogenates (Megaw and Robertson, 1974; Kaufman and Wong,

Figure 1.3 Position and general morphology of the salivary glands. SG, salivary gland; O, ovary; SR, seminal receptacle; MT, Malphigian tubules. i) Type I acini: CC, central cell; PC, pyramidal cell; NC, neck cell; SD, salivary duct. ii) Type II acini: AbIC, abluminal interstitial cell; AdIC, adluminal interstitial cell; A, a-cell; B, b-cell; C, c-cell; Vl, valve; L, lumen. iii) Type III acini: D, d-cell; E, e-cell, F, f-cell. Modified from Megaw, M. W. J. and Beadle, D. J. 1979. Structure and function of the salivary glands of the tick, *Boophilus microplus* Canestrini (Acarina: Ixodidae). International Journal of Insect Morphology and Embryology 8:67-83 with permission from Pergamon Press Ltd.


i
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1983), and exogenously applied catecholamines stimulate fluid secretion both *in vivo* (Kaufman and Phillips, 1973) and *in vitro* (Kaufman, 1976). Of the catecholamines tested, dopamine is the most potent agonist, showing a maximum effect *in vitro* at < 10 µM for at least 8 ixodid species (Kaufman, 1977; Sauer *et al.*, 1979). Dopamine stimulates fluid transport through a dopamine D_1 receptor subtype, that is linked to the activation of adenylate cyclase through a G-protein. A dopamine sensitive adenylate cyclase has been demonstrated (Schmidt *et al.*, 1982; Hume *et al.*, 1984) and the dose-response curve for dopamine on adenylate cyclase activity *in vitro* is similar to that for fluid transport in intact salivary glands (Schmidt *et al.*, 1982). Specific D_1 receptor antagonists, such as thioridazine, and (+) butaclamol are effective inhibitors of salivary gland adenylate cyclase activity and fluid transport (Schmidt *et al.*, 1981; Kaufman and Wong 1983).

Development of Fluid Secretory Competence:

Salivary gland function is also dependent on the stage of feeding of the adult. Although adult males secrete little fluid throughout the entire feeding cycle (Kaufman, 1990), adult females develop a high fluid secretory capacity. Initially, the salivary glands are only able to secrete fluid at a rate of a few nl/min (Kaufman, 1976). As feeding progresses, the salivary glands undergo an extensive intracellular reorganization (Meredith and Kaufman, 1973; Fawcett *et al.*, 1981; Coons and Kaufman, 1988). This results in the development of an extensive fluid secretory labyrinth between the abluminal interstitial, adluminal interstitial, and the F-cell within the type III acini. As feeding continues and development of this fluid secretory labyrinth progresses, fluid secretory competence of the tissue also increases, and reaches a maximum by the end of the slow feeding phase (220 nl/min in *D. andersoni*; Kaufman, 1976).

Salivary Gland Degeneration:

Once the female engorges, the type II and type III acini (and possibly the type I acini in *Haemaphysalis longicornis*; Yanagawa *et al.*, 1987) are resorbed within 3-4 days. Ultrastructurally, this process is characterized by the presence of autophagic vacuoles throughout the degenerating tissue. Using implantation experiments, Harris and Kaufman (1981) demonstrated that this degeneration is triggered by a haemolymph borne factor, that they called 'tick salivary gland degeneration factor' (TSGDF). Presently, the ecdysteroids ecdysone (E) and 20-hydroxyecdysone (20-OHE) are the prime candidates for TSGDF because: (1) Ecdysteroids mimic the physiological effects of TSGDF when glands are exposed to these compounds *in vivo* (Harris and Kaufman, 1985) or *in vitro* (Harris and Kaufman, 1985; Lindsay and Kaufman, 1988). (2) 20-OHE induces the formation of autophagic vacuoles in the fluid secretory labyrinth of the type III acini *in vitro* (Lindsay and Kaufman, 1988). (3) Haemolymph ecdysteroid titers in *A. hebraeum* increase markedly during the first few days post-engorgement (Connat *et al.*, 1985; Kaufman, 1991); this increase correlates well with degeneration of the type II and III acini of the salivary glands (Kaufman, 1991).

Release of TSGDF depends on the degree of engorgement of the female and is probably mediated by abdominal stretch because severing the neural connections between the abdomen and the synganglion (CNS) prevents salivary gland degeneration (Harris and Kaufman, 1984). The size, or critical weight, when TSGDF release occurs is approximately 10x the unfed weight in the two species examined (*A. hebraeum*: 300-400 mg, Harris and Kaufman, 1984; and *A. americanum*: 50-60 mg, Lindsay and Kaufman, 1988).

Associated with the degeneration of the fluid secretory labyrinth in the type III acini is a concurrent reduction in fluid secretory competence of the salivary glands (Harris and Kaufman, 1984). Based on this reduction, a simple, quantitative assay to monitor salivary gland function was developed by Harris and Kaufman (1984). Using this assay, they determined the rate of fluid transport of salivary glands as a function of time post-removal for mated female *A. hebraeum* above and below the critical weight. By day 4 post-removal, mated females above the critical weight lost virtually all fluid secretory competence and autophagic vacuoles were prevalent throughout the type III acini (Harris and Kaufman, 1984). Although mated females below the critical weight also lost about 75% of their secretory competence, this loss does not reflect tissue degeneration because: (1) They retained this level of secretory competence for at least 11 more days. (2) If the females were allowed to reattach to the host, much of the fluid secretory competence was regained within 2 days. (3) There were no autophagic vacuoles within the type III acini of ticks below the critical weight (Harris and Kaufman 1984). The cause of this reversible loss of secretory competence is unknown, although Kaufman (1986) provided some evidence to suggest an uncoupling of the dopamine receptor from the adenylate cyclase system.

Although the above results show that a loss of fluid secretory competence is an easily measured index of salivary gland degeneration[1], fluid transport alone cannot be used as a definitive indicator of gland degeneration. One must also demonstrate the presence of autophagic vacuoles within type III acini.

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[1] Hereafter, 'salivary gland degeneration' is defined as the loss of fluid secretory competence of the salivary glands. It should be noted that only type II and type III acini of the salivary gland degenerate once feeding is complete. Type I acini do contain autophagic vacuoles fro at least several weeks after engorgment (exceptions include *H. longicornis*; Yanagawa *et al.*, 1987). Furthermore, although the loss of fluid secretory competence of the salivary gland is correlated with resorption of the fluid secretory labyrinth within the type III acini, true degeneration must be confirmed by the presence of autophagic vacuoles within the type II and type III acini.

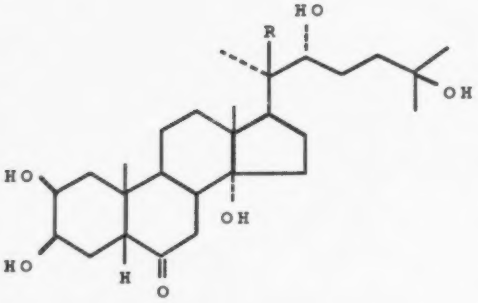
Endocrinology:

Although it is evident that fundamental processes such as embryonic development (Solomon *et al.*, 1982; Connat *et al.*, 1984; Wigglesworth *et al.*, 1985), moulting (Cox, 1960; Mango *et al.*, 1976; Diehl *et al.*, 1986), salivary gland function (Harris and Kaufman, 1981; Coons and Kaufman, 1988), and ovary development (Lunke and Kaufman 1993) are controlled by endocrine processes, as yet the only class of hormones positively identified in ticks are the ecdysteroids (Delbecque *et al.*, 1978). Ecdysteroids are a class of molecule found in virtually every arthropod examined so far, as well as several non-arthropod groups (table 1.1). Ecdysteroids are derived from cholesterol. They possess the full C27 carbon skeleton of cholesterol, exhibit a cis fusion (5β-H) of the A and B rings, have two functional moieties in the B ring (a 6-keto-7-ene system) and possess several hydroxyl groups at various positions, the latter rendering these molecules appreciably water-soluble (figure 1.4). They differ from vertebrate steroids primarily by the retention of the 8 carbon side chain attached at C17, the cis A:B ring configuration with a 5β-H attachment at C5 (vs a trans A:B ring configuration for vertebrate steroids), and the multiple hydroxylations. These differences between the two families of steroids ensure that no functional crossover exists. The two primary active ecdysteroids, ecdysone (E) and 20-hydroxyecdysone (20-OHE), were first identified in ticks by Delbecque *et al.* (1978). In both larval and nymphal moults, an increase in E titre has been temporally correlated with apolysis, with the peak in ecdysteroid titre occuring at the time of epicuticle deposition (Diehl *et al.*, 1982; Dotson *et al.*, 1991). Injection of 20-OHE into unfed males is able to stimulate an increase in the number of DNA synthesizing germ cells, suggesting an important role of ecdysteroids in

Table 1.1 Identification of ecdysteroids in various invertebrate phyla. Modified from Horn and Bergamasco (1985).

Phylum	Class	Reference or Review
Arthropoda	Insecta	Butenandt and Karlson, 1954; Horn and Bergamasco, 1985; De Loof *et al.*, 1984; Spindler *et al.*, 1980; Spindler *et al.*, 1984.
	Malacostraca	Bebbington and Morgan, 1977; Lachaise and Hoffmann, 1977.
	Merostomata	Jegla and Costlow, 1979.
	Arachnida	Bonaric and De Reggi, 1977; Delbecque *et al.*, 1978; Diehl *et al.*, 1986.
	Pycnogonida	Behrens and Buckmann, 1983; Buckmann *et al.*, 1986.
Cnidaria		Guerriero and Pietra, 1985.
Platyhelminthes	Cestoda Trematoda	Rees and Mendis, 1984; Koolman *et al.*, 1984; Mercer *et al.*, 1987; Nirde *et al.*, 1984; Rees and Mercer, 1986.
Nematoda		Rees and Mendis, 1984; Koolman *et al.*, 1984; Nirde *et al.*, 1984; Rees and Mercer, 1986.
Mollusca	Gastropoda	Takemoto *et al.*, 1967; Romer, 1979; Whitehead and Selheyer, 1982.
Annelida	Oligochaeta Hirudinea	Porchet *et al.*, 1984; Sauber *et al.*, 1981.
Echinodermata		Karlson, 1983.

Figure 1.4 Chemical structures of the active ecdysteroid hormones ecdysone and 20-hydroxyecdysone. Also shown is cholesterol (the precursor of all ecdysteroids) and testosterone (a typical vertebrate Steroid). Carbon skeletal numbering system is indicated on the cholesterol molecule.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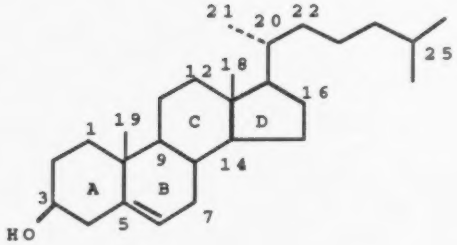
R=H Ecdysone
R=OH 20-Hydroxyecdysone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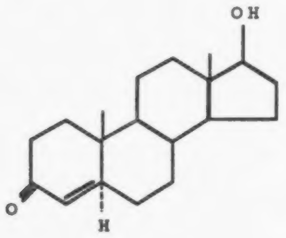
Cholesterol



Testosterone

spermatogenic induction (Oliver, 1992). In females, ecdysteroid titres increase only after feeding has begun and the last 3 days of feeding are characterized by a dramatic increase in the quantity of RIA positive material in whole tick extracts (Connat *et al.*, 1985). This increase is tissue specific because haemolymph and ovary ecdysteroid titres remain low until after feeding is complete (Connat *et al.*, 1985); the location of this ecdysteroid pool in whole extracts is not yet known. Once the female engorges, she detaches from the host and haemolymph ecdysteroid titre increases within 2-3 days. This is followed by an increase in ovarian ecdysteroid concentration that ultimately surpasses that of the haemolymph (Kaufman, 1991). During this time, the salivary glands degenerate, vitellogenesis begins, and ovary weight increases. During oviposition, ecdysteroids in the form of E and 20-OHE (as in *A. hebraeum*; Connat *et al.*, 1985; Connat and Dotson, 1988) or apolar conjugates (as in *B. microplus* and *Rhipicephalus appendiculatus*; Crosby *et al.*, 1986) accumulate in the eggs. Although their exact role is not clear, Wigglesworth *et al.* (1985) noted the occurrence of distinct hormone 'peaks' during *B. microplus* embryogenesis and suggested that these ecdysteroid peaks are important for the deposition of embryonic cuticle, as is the case in *Locusta migratoria* (Lagueux *et al.*, 1979).

Although E and 20-OHE are found in the haemolymph and ovarian tissue, these tissues also contain substantial amounts of polar and apolar conjugates, possibly representing ecdysteroid precursors or inactive ecdysteroid conjugates. Connat *et al.* (1987) showed that injected [^{3}H]-20-OHE was metabolized to both polar and apolar products within 6 h of injection. By two days after injection, the radiolabelled 20-OHE had completely disappeared and the radioactivity was redistributed between several apolar and polar products, with one distinct polar product representing 30% of the total radioactivity in the carcass. Eight days after injection, the greatest proportion of radioactivity was associated with apolar products, representing 60% of the total radiolabel in the carcasses. Treatment of this fraction with pig liver esterase revealed this fraction to consist of conjugates of 20-OHE (39%) and the 3-epimer of 20-OHE (61%; Connat *et al.* 1987).

Although it is apparent that ticks synthesize ecdysteroids, the tissue source(s) remains elusive. Based on ultrastructural evidence, Binnington (1981) suggested the lateral segmental organs (closely associated with the synganglion) as possible candidates for ecdysteroid synthesis. Sonenshine *et al.* (1985), noted significant accumulations of ecdysteroids in the vicinity of the synganglion and associated nerve plexi of feeding *Dermacentor variabilis* and *Hyalomma dromedarii*. Furthermore, autoradiographs taken of adult synganglia and associated nerve plexi from ^{14}C-cholesterol-inoculated nymphs demonstrated substantial incorporation of ^{14}C into these tissues. It was not determined, however, if this accumulation of ecdysteroid or ^{14}C-labeled product was due to tissue synthesis or tissue storage. Schriefer *et al.* (1987) implicated fat body as the endocrine source of ecdysteroids in *Dermacentor variabilis*. A later study by Zhu *et al.* (1991) demonstrated that, in the argasid tick *Ornithodoros parkeri*, the endocrine source of ecdysteroids is the epidermis, while the fat body is responsible for the conversion of E to 20-OHE. Although Oliver (1992) suggests that this may also be the case in ixodid ticks, the source(s) of ecdysteroids in ixodid ticks still remains unclear.

Male Derived Mating Factors:

Although, so far ecdysteroids are the only hormones positively identified in ticks, other hormones/factors have been postulated. These include several male derived 'mating factors' such as: (1) A chemical factor derived from within the endospermatophore that initiates the rapid expansion phase of feeding (Pappas and Oliver, 1972). (2) A 12.5 kD protein from the accessory gland is responsible for initiating capacitation of prospermia (Shepherd *et al.*, 1982). (3) A high molecular weight protein factor secreted by the spermatozoa after capacitation is completed and responsible for stimulating oviposition (Germond and Aeschlimann, 1977; Sahli *et al.*, 1985; Connat *et al.*, 1986). (4) A chemical factor derived from the male genital tract that prevented the normal time course of salivary gland degeneration in engorged female *A. hebraeum* (Harris and Kaufman, 1984).

Objectives of this Thesis:

Kaufman and Harris (1983) observed that the salivary glands of virgin females above the critical weight did not degenerate like those of mated females. While investigating the mechanisms responsible for salivary gland degeneration, Harris and Kaufman (1984) examined the possible signals that mating could provide the female, and cue the salivary gland degenerative process. This could include a neural signal due to seminal receptacle stretch caused by the endospermatophore, a chemical signal derived directly from the spermatophore or endogenously from the seminal receptacle itself, or a combination of both. Cutting all seminal receptacle innervation in mated, engorged females had no effect on salivary gland degeneration, whereas complete removal of the seminal receptacle prevented salivary gland degeneration. Replacement of the seminal receptacle with a male genital tract homogenate permitted salivary gland degeneration to occur. Harris and Kaufman (1984) thus concluded that a ‘male factor’ is transferred to the female during mating. They did not examine the chemical nature of this factor, nor the mechanism by that it acts. This thesis examines male factor in the ixodid tick, *Amblyomma hebraeum* (Koch), with respect to the following: (1) Is male factor an absolute requirement for salivary gland degeneration, or does male factor have primarily a modulating role? (2) Where within the male genital tract is male factor produced? (3) What other tissues have male factor activity? (4) What are some of the basic chemical properties of male factor, and can they be used to distinguish male factor from other tick factors? (5) How does male factor influence salivary gland degeneration?

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Chapter 2

The Influence of Male Factor on Salivary Gland Degeneration[2]

Introduction:

Female ixodid ticks take an enormous blood meal over their 7-10 day feeding period, often increasing their weight by as much as 100-fold (Balashov, 1972). As feeding progresses, the nutrient portion of the meal is concentrated in the gut by the elimination of excess fluid back into the host by salivation (Tatchell, 1967; Kaufman and Phillips, 1973a).

Salivary gland function depends on the degree of engorgement. In unfed *Dermacentor andersoni*, the salivary glands are able to secrete fluid at only a few nl/min (Kaufman, 1976). As feeding progresses their fluid secretory competence increases and eventually reaches a maximum of 200-250 nl/min prior to engorgement (Kaufman, 1976). Once the female engorges and detaches from the host, the salivary glands degenerate within 3-4 days, a process triggered by the release of a 'tick salivary gland degeneration factor' (TSGDF; Harris and Kaufman, 1981). Release of TSGDF is likely triggered by abdominal stretch (Kaufman, 1983) and occurs at a 'critical weight' of approximately 10 times the unfed weight (Harris and Kaufman, 1984; Lindsay and Kaufman, 1988). In *Amblyomma hebraeum* this critical weight is about 300-400 mg.

Mating is an important prerequisite for the normal engorgement of ixodid ticks and degeneration of their salivary glands. Although both the male and female must feed for a few days before mating to allow for genital tract maturation (Khalil, 1970; Feldman-Muhsam and Borut, 1971; Norval and Capitini, 1974), mating usually occurs prior to the female reaching the critical weight. Most females that do not mate will not feed beyond the critical weight and the feeding cycle can be extended to over 40 days (Pappas and Oliver, 1971). Of the few virgin females that do obtain a size above the critical weight, the salivary glands do not degenerate within 3-4 days after detachment as occurs in their mated counterparts (Kaufman, 1983). Thus, mating affects both feeding and salivary gland degeneration.

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[2] A version of this chapter (see appendix 1) has been published as Lomas and Kaufman. 1992a. Journal of Insect Physiology 38:595-604.

During mating, the male deposits a spermatophore onto the ventrum of the female that is then internalized and stored in the female's seminal receptacle (Moorhouse, 1966; Feldman-Muhsam *et al.*, 1973). Harris and Kaufman (1984) demonstrated that in *A. hebraeum*, surgical removal of the seminal receptacle from mated females above the critical weight could prevent salivary gland degeneration. Salivary gland degeneration could be restored either by implanting the seminal receptacle into the haemocoel, or by injecting a homogenate of male gonad. Harris and Kaufman (1984) thus demonstrated that in mated females, a male derived chemical factor, which they called 'male factor', is responsible for promoting salivary gland degeneration within 3-4 days of detachment.

In this study, I demonstrate a relationship between mating and the normal time course of feeding, salivary gland degeneration, and initiation of oviposition. I also establish the primary location of male factor activity within the male genital tract and identify other tick tissues that contain male factor activity.

Materials and Methods:

Ticks:

Adult *A. hebraeum* were obtained from a laboratory colony maintained in darkness at 26°C and 95% relative humidity. Adult ticks were confined to the backs of rabbits (Flemish Large x giant Lop Eared) as previously described by Kaufman and Phillips (1973b). To rear mated females, equal numbers of males and females were confined together. To rear virgin females, males were first treated by plugging their genital pore with a drop of glue (cyanoacrylate compound; Loctite Super Bonder 495, Loctite Corp., Newington, Conn., USA) before confining them with the females. Once all females had attached (12-24 h after placement) all males were removed. Females were later confirmed as virgin by the absence of spermatozoa in a smear of the seminal receptacle. The weights of ticks used in this study are as follows: females below the critical weight = 150-250 mg, females above the critical weight = 400-1200 mg.

Assay for Salivary Gland Secretory Competence:

The fluid secretory competence of salivary glands was determined according to the method of Harris and Kaufman (1984). Salivary glands were excised from ticks and the main salivary duct ligated with a fine strand unraveled from 8-0 surgical silk thread. The salivary glands were then transferred to tissue culture medium 199 (TCM 199; Gibco Inc., Grand Island, New York) without sodium bicarbonate, buffered with 10 mM 3-[N-morpholino]-propanesulphonic acid (MOPS; Sigma, St Louis, Mo., U.S.A.) and osmotically adjusted to 360 mosmol l^{-1} by adding 2.1 gm l^{-1} NaCl. The pH was adjusted to 7.3 using NaOH (see appendix 2). At the time of assay, salivary glands were removed from the TCM 199, blotted gently to remove excess medium and the wet weights were measured to the nearest 10 µg on a Sartorius 2474 microbalance. Salivary glands were then incubated in constantly stirring TCM 199 containing 10 µM dopamine (Sigma) that elicits a maximal rate of fluid transport (Kaufman, 1976; Harris and Kaufman, 1984). After a 10 or 15 minute incubation period, salivary glands were removed, blotted, and reweighed. The net weight increase was taken as an index of fluid secretory competence. All fluid transport rates were normalized to mg/gland/10 min.

Male Factor Bioassay:

To test a tissue for male factor activity, the tissue was homogenized in ice cold 1.2% NaCl, centrifuged, and injected into virgin females above the critical weight on day 0 post-removal. Virgin ticks were then incubated at 26°C, 95% relative humidity until day 4 post-removal, at that time the fluid secretory competence of their salivary glands was determined. Controls consisted of mated and virgin ticks above the critical weight, each injected with 1.2% NaCl. High fluid transport rates indicated a lack of male factor activity while low fluid transport rates indicated the presence of male factor activity.

Injection of Tissue Homogenates:

Tissues from donor ticks were excised and homogenized as described above. The homogenates were then centrifuged at approximately 8000 **x g** for 5 min and the supernatant (= crude extract) injected into virgin females (either 20 or 25 µl injected volume/female, as indicated) using a 100 µl Hamilton syringe equipped with a 30 gauge needle modified so as to reduce the residual volume (see appendix 3). Injections were made into the haemocoel through the articulation between the capitulum and the scutum. Controls consisted of injecting an equal volume of 1.2% NaCl. Tissues included: (a) male genital tract (either 1 or 2 genital tract equivalents/25 µl) from unfed or fed males, (b) the accessory glands (2 male equivalents/20 µl), (c) reproductive tract with ejaculatory duct (2 male equivalents/20 µl), (d) salivary glands (1 pair/ 20 µl), (e) ovary from fed, mated females (1 ovary/20 µl),

(f) the synganglion from either fed males or females (4 synganglia/20 µl).

Density Gradient Separation:

Reproductive tract homogenates were layered over a discontinuous sucrose gradient consisting of two layers, 1 ml of a 40% sucrose solution (w/w; density = 1.18 gm/ml) and 1.5 ml of a 47% sucrose solution (w/w; density = 1.21 gm/ml). The gradient was centrifuged for 30 min in a Beckman L2-65B ultracentrifuge equipped with a SW 55 Ti rotor at an average of 21,300 x **g**. After centrifugation, 3 fractions could be identified and are referred to as top (density < 1.18 gm/ml), middle (density = 1.18-1.21 gm/ml), and bottom (density >1.21 gm/ml) fractions. In this system, spermatozoa migrate to the bottom fraction. Each fraction was removed, washed 3 times in 1.2% NaCl, and injected into virgin females on day 0 post-removal (final concentration of derived fraction = 2 male reproductive tract equivalents/ 20 µl NaCl) and male factor activity assayed as described above. All protein measurements were made using the method of Lowry *et al.* (1951)

Statistics:

All data are reported as mean ± standard error mean (N). Statistical significance was determined as followed: Data were first analyzed for normality using an F-Test. For all data that showed a normal distribution, statistical significance was determined by one way analysis of variance (ANOVA) using the MIDAS statistical package of the University's main frame computer. For data that did not show normality, statistical significance was determined using the Mann-Whitney U nonparametric test (using Statview™ SE+Graphics, Abacus Concepts Inc., California, USA) on a Macintosh computer.

Results:

Feeding Response of Virgin Females:

Mated females typically feed for 7-8 days and then drop from the host. Virgin females can remain attached to the host for at least 45 days (figure 2.1). During this time, the average weight increase of virgins was significantly less than that of mated females that had fed for only 7.5 days (355 ± 80 mg, N=16 for virgins vs 2230 ± 260 mg, N=6 for mated females; $p<0.001$). The weight distribution of virgin females was bimodal, with a small proportion of females feeding well above the critical weight (740 ± 100 mg N=4; figure 2.1) by 21 days of feeding, while the majority remained at 180 ± 20 mg (N=15). No significant increase in body weight occurred thereafter in both samples of virgins.

With prolonged feeding periods, rabbits are able to mount an immune response against ticks (reviewed by Wikel and Ramchandra, 1992). In an attempt to determine if an immune response is responsible for inhibiting virgin females from feeding, virgins were transferred from the initial host to a naive host after a 10 day feeding period (table 2.1). Virgin females fed for 10 days on an initial host significantly increased in weight from 30.8 ± 0.3 mg to 202 ± 20 mg (N=44; $p<0.001$), with 10% of those females surpassing the critical weight. After the initial 10 day feeding period, those females below the critical weight were divided into two groups; one group was placed back on the initially challenged host, and the second group was placed on a fresh, naive host. Ticks returned to the initial host for an additional 10 days increased in body weight from 157 ± 20 mg (N=19) to 241 ± 51 mg (N=12), with 21% of those females reaching a size above the critical weight (472 ± 27 mg, N=4). Ticks fed for the remaining 10 days on a naive host also showed a significant increase in weight (153 ± 21 mg, N=19 to 292 ± 71 mg, N=10; $p<0.001$). Of these virgins, 16% reached a size above the critical weight (594 ± 96 mg). There was, however, no

Figure 2.1 Feeding rates of mated or virgin adult, female *Amblyomma hebraeum*. Mean ± SEM. Where no bar appears, SEM is smaller than symbol size.

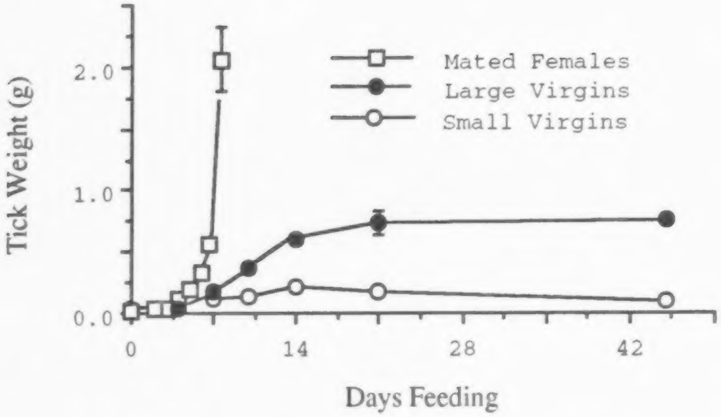

Tick Weight (g)
2.0
1.0
0.0
0
14
28
42
Days Feeding
Mated Females
Large Virgins
Small Virgins

Table 2.1 The effect of feeding partially fed virgin females on previously challenged vs naive rabbits. Initial and final weights (N) are given for virgin females fed initially on a naive rabbit for 10 days, then transfered to either the same rabbit (= challenged) or a new rabbit (= naive) for a second 10 day feeding period.

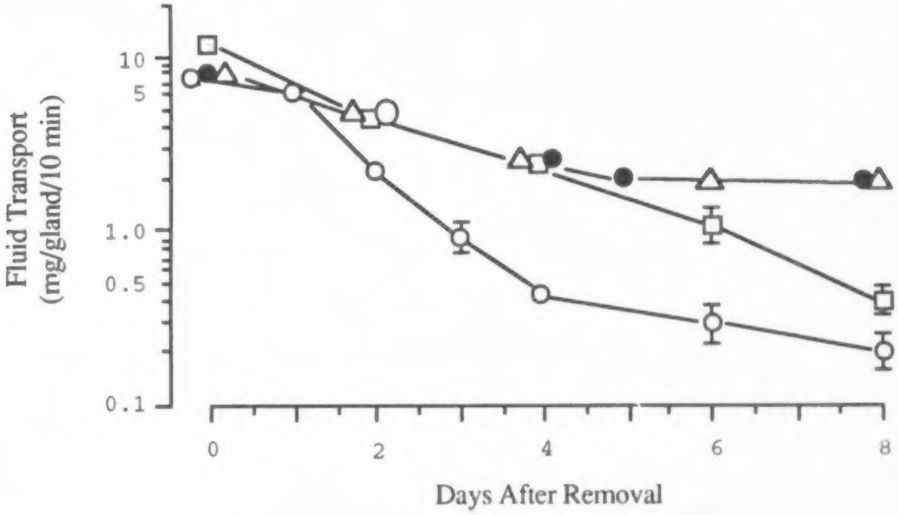
Treatment	Initial tick weight (mg)	Final tick weight (mg)	Dead	Final tick weight as a function of the critical weight	
				below critical weight	above critical weight
First tick feed (naive rabbit)	30.8 ± 0.3 N=60	202 ± 20 N=44	- N=16	162 ± 14 N=38	457 ± 43 N=6
Second tick feed (challenged rabbit)	157 ± 20 N=19	241 ± 51 N=12	- N=7	130 ± 25 N=8	472 ± 27 N=4
Second tick feed (naive rabbit)	153 ± 21 N=19	292 ± 71 N=10	- N=9	163 ± 33 N=7	594 ± 96 N=3

significant increase in overall weight (p=0.76) or % engorgement (p=0.74) between ticks fed on the initially challenged host or the naive host for the final 10 days of feeding.

Time Course of Salivary Gland Degeneration:

The time course of salivary fluid secretory competence in virgin females and mated females above and below the critical weight is shown in figure 2.2. Mated females above the critical weight lost 95% of their fluid secretory ability by 4 days post-removal. Mated females below the critical weight, on the other hand, lost 71% of their secretory capability by 4 days post-removal and maintained this secretory level for at least 4 more days. The time course of fluid secretory competence for virgin females below the critical weight was essentially identical to that of comparably sized mated females. The time course of fluid secretory competence for virgin females above the critical weight was like that of mated females below the critical weight up to day 4 post-removal (2.18 ± 0.24 mg/gland/10 min for virgins and 2.27 ± 0.19 mg/gland/10 min for mated females); this level was significantly higher than that of mated females above the critical weight (0.39 ± 0.07; $p<0.001$). The secretory competence of the salivary glands from virgin females, however, did not then plateau as in females below the critical weight, but instead decreased further and by day 8 attained a level (0.44 ± 0.07 mg/gland/10 min) not significantly different from that of day 4 or day 8 mated females above the critical weight ($p>0.05$). It thus appears that in virgins above the critical weight, salivary gland degeneration is not completely inhibited as thought earlier (Harris and Kaufman, 1984), but is delayed by 3-4 days.

Figure 2.2 Fluid transport rate of salivary glands as a function of time post-removal. Mean ± SEM. Where no bar appears, SEM is smaller than the symbol size. ○, mated females above the critical weight; ●, mated females below the critical weight; □, virgin females above the critical weight; △, virgin females below the critical weight


Fluid Transport
(mg/gland/10 min)
10
5
1.0
0.5
0.1
0
2
4
6
8
Days After Removal

Effect of Vehicle Injections:

To assess the male factor activity of various tissues, homogenates were prepared in a 1.2% NaCl. I initially tested to see if this solution has any effects on the fluid secretory competence of the salivary glands. The injection of ice cold 1.2% NaCl caused a non-significant increase in the fluid secretory competence of salivary glands in mated females, above ($p=0.07$) and below ($p=0.14$) the critical weight (figure 2.3) by day 4 post-injection. Injection of NaCl into virgin females, however, did cause a significant increase in fluid secretory competence ($p=0.22$). All fluid transport rates increased as follows: (1) Virgins above the critical weight, 2.12 ± 0.34 (N=17) to 3.24 ± 0.29 (N=30) mg/gland/10 min. (2) Mated females below the critical weight, 2.28 ± 0.19 (N=14) to 2.88 ± 0.31 (N=19) mg/gland/10 min. (3) Mated females above the critical weight, 0.21 ± 0.06 (N=11) to 0.72 ± 0.24 (N=13) mg/gland/10 min. Injection of 1.2% NaCl did not change overall trends between virgin and mated females (figure 2.3).

Location of Male Factor Within the Male Genital Tract:

I next tested the ability of crude extracts of male genital tract to hasten salivary gland degeneration in virgin females above the critical weight. Such females, when injected with a homogenate of male genital tract showed a significant decrease in fluid secretory competence as compared to virgin controls (figure 2.4). This decrease occurred in a dose dependent manner, and with the injection of two male genital tract equivalents, the fluid secretory competence (0.87 ± 0.15 mg/gland/10 min; N=15) was not significantly different from that of the mated controls (0.68 ± 0.24 mg/gland/10 min; N=18; $p>0.05$). Injection of three male genital tract equivalents did not significantly reduce the rate of fluid transport (0.84 ± 0.34 mg/gland/10 min; N=8) below that of two genital tract equivalents or the mated controls.

Figure 2.3 The effect of 1.2% NaCl injection on the fluid transport rate of salivary glands taken from virgin or mated females. Females were injected with NaCl at the time of removal and their salivary glands assayed 4 days later. NaCl = injected females; Control = non-injected females. Mean ± SEM.

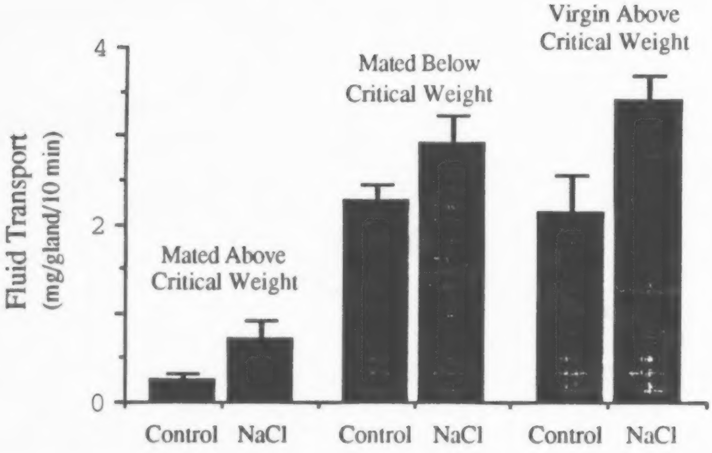
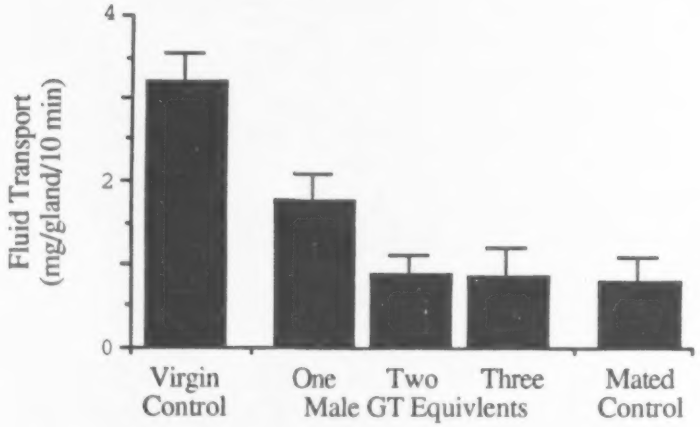

Fluid Transport
(mg/gland/10 min)
4
2
0
Mated Above
Critical Weight
Mated Below
Critical Weight
Virgin Above
Critical Weight
Control
NaCl
Control
NaCl
Control
NaCl

Figure 2.4 Male factor content of the genital tract from fed males. The fluid transport rate of salivary glands from virgin females above the critical weight was determined four days after the injection of one, two, or three male genital tract equivalents. Virgin controls and mated controls each received veichle injection. Mean ± SEM.


Fluid Transport
(mg/gland/10 min)
4
2
0
Virgin
Control
One
Two
Three
Male GT Equivlents
Mated
Control

The male genital tract is composed of two major parts, the accessory gland portion and the male reproductive tract, including the testis and vas deferens, all of that join together at the ejaculatory duct (see figure 1.2). I next tested these two areas for male factor activity. The reproductive tract and ejaculatory duct were separated surgically from the accessory gland portion. Male factor activity occurred exclusively within the male reproductive tract (figure 2.5). The fluid secretory competence of virgin females above the critical weight injected with this homogenate was not significantly different from the mated controls (experimental, 1.10 ± 0.16, N=16 vs control, 1.06 ± 0.13, N=10, mg/gland/ 10 min; $p>0.05$; figure 2.5). The accessory glands, on the other hand, contained no detectable male factor activity (figure 2.5; $p>0.05$).

Further localization of male factor within the reproductive tract was achieved using the discontinuous sucrose density gradient centrifugation procedure of Dawson and Scott (1964). This technique can separate the spermatozoa of ticks (density > 1.21 g/ml) from the seminal and testicular fluids and other tissues (density < 1.21 g/ml). Most male factor activity was associated with the middle fraction (density between 1.18-1.21 g/ml), and some with the top fraction (density < 1.18 g/ml) but no activity was associated with the bottom (spermatozoa) fraction (figure 2.6).

As the volume of various fractions injected into virgins was standardized to 'reproductive tract equivalents' and not to the amount of protein present, the apparent lack of male factor activity seen in the bottom, and the reduced activity seen in the top fractions could have resulted from vastly unequal doses of male factor. The protein content of all injected fractions was measured and is shown plotted against the effect they induced on salivary gland fluid transport (figure 2.7). Injection of varying protein amounts of the middle fraction caused a dose dependent decrease in fluid

Figure 2.5 Localization of male factor activity in the male genital tract. Fluid transport rate of salivary glands taken from virgin females above the critical weight was determined four days after injecting either a testis-vas deferens homogenate (Reprod) or an accessory gland homogenate (Accessory). Virgin or mated controls received veichle injection. Mean ± SEM.

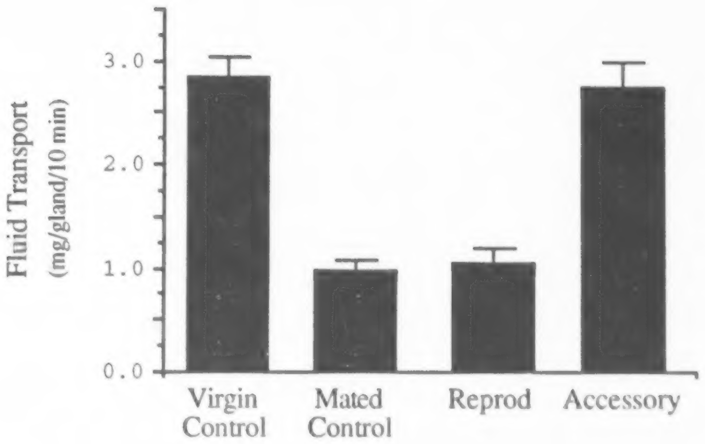
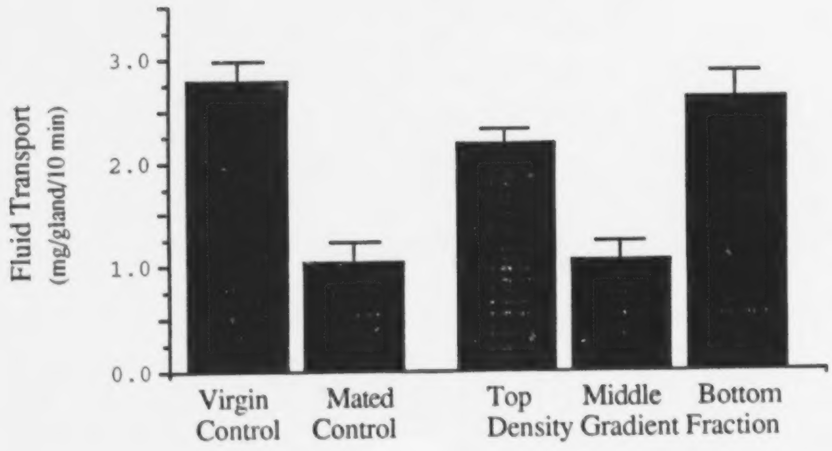
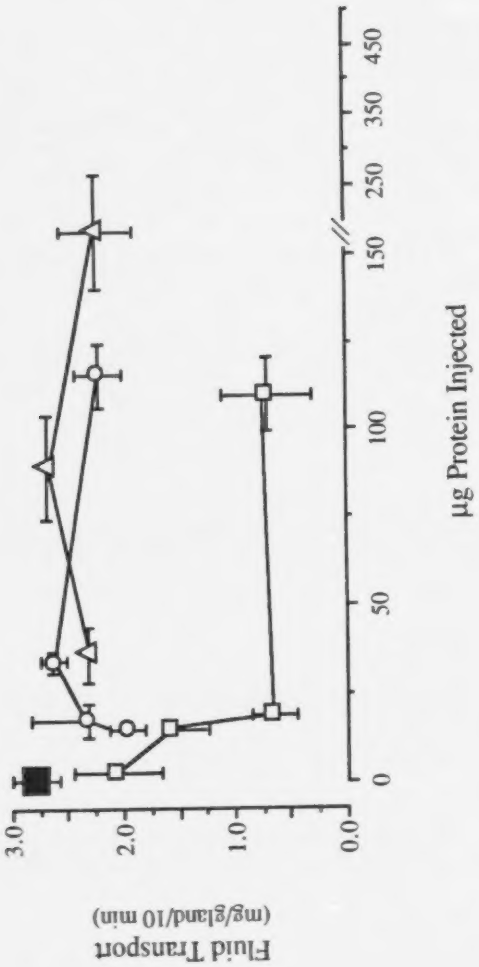

Fluid Transport
(mg/gland/10 min)
3.0
2.0
1.0
0.0
Virgin Control
Mated Control
Reprod
Accessory

Figure 2.6 Male factor activity in three fractions of a reproductive tract homogenate separated by discontinuous sucrose density gradient centrifugation. Top fraction, middle fraction, and bottom fraction (= spermatozoa) have relative densities of <1.18, 1.18-1.21, and >1.21 gm/ml. Virgin and mated controls each received injection of veichle alone. Mean ± SEM.



Fluid Transport
(mg/gland/10 min)
3.0
2.0
1.0
0.0
Virgin Control
Mated Control
Top Density
Middle Gradient
Bottom Fraction

Figure 2.7 Protein content of the top, middle, and bottom fractions of a male genital tract homogenate separated on a discontinuous sucrose density gradient and plotted as a function of fluid transport 4 days after injection. ○, top fraction; □, middle fraction; △, bottom fraction; ■, Control. Mean ± SEM.


Fluid Transport
(mg/gland/10 min)
3.0
2.0
1.0
0.0
0
50
100
150
250
350
450
µg Protein Injected

transport, a maximal effect occurring with the injection of as little as 19 ± 2 µg protein (N=3). In this case, fluid transport was reduced from 2.79 ± 0.21 (N=11; see figure 2.7) to 0.90 ± 0.19 (N=3) mg/gland/10 min. Conversely, injection of as much as 184 µg protein of the bottom fraction or 116 µg protein of the top fraction was not able to reduce fluid transport rates significantly. It therefore appears that the discontinuous density gradient is able to adequately separate male factor from other reproductive tract components and this activity is not associated with the spermatozoa. Male factor is associated with some other tissue of the male reproductive tract, or perhaps the testicular fluid.

Effect of Feeding on Male Factor Activity:

As mentioned earlier, before males will mate they must feed for several days to stimulate the final stages of genital tract maturation. I tested the effect of this feeding period on male factor activity. Figure 2.8 shows that feeding enhances male factor activity within the male reproductive tract. Male factor extracts prepared from fed males induced an additional 55% decrease in fluid transport as compared to extracts prepared from unfed males.

Male factor activity in other tissues:

To determine if male factor is a gonad specific substance, extracts of other tick tissues were also tested for male factor activity. These included male salivary gland, the synganglia of both fed males and females, and the ovary from fed mated females. Male salivary gland tissue contained no detectable male factor activity (3.04 ± 0.49 mg/gland/10 min, N=12 for salivary glands vs 2.80 ± 0.53 mg/gland/10 min, N=13 for virgin controls; $p > 0.05$; figure 2.9). The synganglion from either fed males or females, or the ovary from fed females showed a small but significant amount of male factor like activity ($0.05 > p > 0.01$).

Figure 2.8 Effect of feeding on the male factor content in the male reproductive tract. Fluid transport rate of salivary glands taken from virgin females above the critical weight 4 days after injection with a homogenate of two male genital tracts from unfed males or males fed for five days. Virgin and mated controls are injected with veichle only. Mean ± SEM.

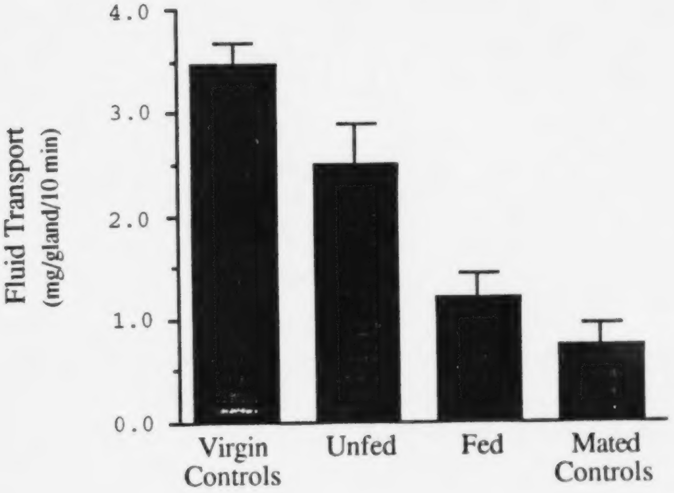
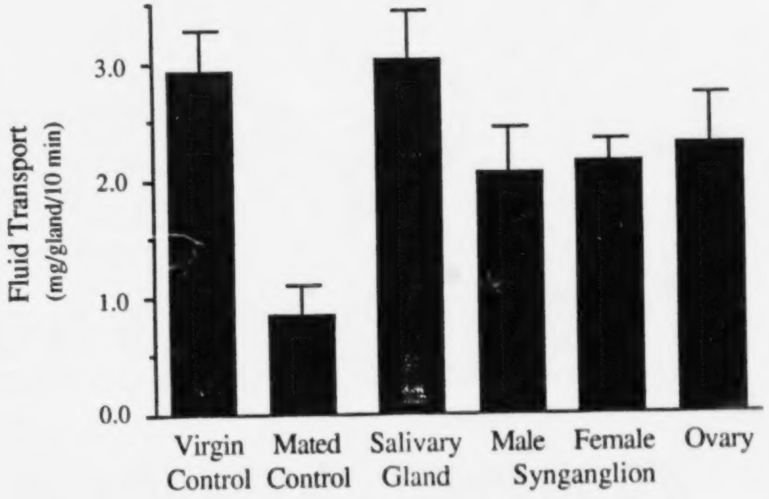

Fluid Transport
(mg/gland/10 min)
4.0
3.0
2.0
1.0
0.0
Virgin Controls
Unfed
Fed
Mated Controls

Figure 2.9 Male factor activity in various tick tissues. Fluid transport rate of salivary glands taken from virgin females above the critical weight 4 days after injection with homogenates of either salivary gland, male or female synganglion, or ovary from mated females above the critical weight. Virgin and mated controls received veichle injection only. Mean ± SEM.


Fluid Transport
(mg/gland/10 min)
3.0
2.0
1.0
0.0
Virgin Control
Mated Control
Salivary Gland
Male
Female
Synganglion
Ovary

Male Factor and the Pre-ovipositional Latent Period:

Given the delay in salivary gland degeneration that occurs in the absence of male factor, I tested whether the ovipositional latent period was lengthened in the absence of mating. The ovipositional latency period was determined for both mated and virgin females above the critical weight (table 2.2). Mean ovipositional periods for virgin females (10.8 ± 0.9 days) was not significantly longer than that of mated females (10.1 ± 0.4 days; p=0.38).

Table 2.2 The influence of mating on the ovipositional latent period in engorged female *Amblyomma hebraeum*.

	Sample Size (N)	Tick Weight (g) mean ± SEM	Day of Oviposition mean ± SEM	P - value
Mated	11	1.58 + 0.14	10.09 + 0.37	0.3838
Virgin	6	0.92 + 0.11	10.83 + 0.91	

Discussion:

Adult females feeding in the absence of males do not enter the rapid engorgement phase even after 45 days (figure 2.1). This does not, however, preclude some virgin females from feeding beyond the critical weight, even though the rate at that they feed may be greatly depressed (figure 2.1). Why all virgins do not eventually reach the critical weight is not known. Brown and Askenase (1986) demonstrated that feeding *A. americanum* ticks on guinea pigs challenged by either a previous tick infestation or by injection of salivary gland extracts, caused a significant increase in tick rejection and a significant decrease in average engorged weight. Likewise, Brossard (1992) demonstrated a dramatic decrease in the weights of engorged *Ixodes ricinus* females when fed on previously challenged rabbits. Treatment of these challenged rabbits with the immunosuppressive drug, cyclosporin A attenuated the effects of host resistance, suggesting that cell-mediated immunity plays an important role in depressed feeding rates (Brossard, 1992). Because of the extended feeding periods required to obtain large virgin females, the low numbers of ticks ultimately exceeding the critical weight could be a result of the development of a host immune response. Although host immunity may play an important role in tick feeding, it did not appear to be the primary reason for the depression of feeding in virgins in this study. Although the average weight obtained by virgins fed on two separate naive rabbits was significantly greater than that obtained by virgins that fed on the same host for the entire feeding period, the overall number of virgins that fed beyond the critical weight was not significantly increased (table 2.1).

Following the blood meal, the salivary glands of female ixodid ticks degenerate under the influence of a tick salivary gland degeneration factor (Harris and Kaufman, 1981). When identifying some of the components in the pathway leading to the release of this degeneration factor, Harris and Kaufman (1984) implicated a substance originating in the male gonad that they called male factor. At that time, it was thought that male factor might be an essential component in the total pathway. Here we must modify this view with the demonstration that salivary gland degeneration can proceed in the absence of male factor, although the process is delayed by 3-4 days (figure 2.3). With the further evidence that male factor is also able to hasten the release of ecdysteroids into the haemolymph of large virgins (Lomas and Kaufman, 1992b; see also chapter 4), it appears that male factor is performing a modulatory rather than essential role. Whatever role male factor plays, it is bound to be subtle, and likely a challenge to uncover.

In this study, I found that a dose of two male genital tract equivalents was required to achieve the maximal effect (figure 2.4), whereas Harris and Kaufman (1984) found that one male genital tract equivalent was sufficient. The discrepancy may be due to the fact that here I used virgins (thus never exposed to male factor) while Harris and Kaufman (1984) worked with mated ticks that had been surgically deprived of the seminal receptacle. It now appears that male factor is normally transferred from the seminal receptacle to the haemolymph of mated females (Lomas and Kaufman, 1992b; see also chapter 4). Perhaps these mated, operated females used by Harris and Kaufman (1984) retained some subthreshold amount of male factor within the haemolymph so that a lower exogenous dose was fully effective.

Male factor is found exclusively in the testis/vas deferens portion of the male gonad (figure 2.5), and more specifically it is presumably associated with the testicular fluid. Our evidence for this, although indirect, is that: (1) male factor is not associated with the spermatozoa, as it can be easily separated using a density gradient centrifugation technique (figure 2.6), and (2) male factor activity is markedly increased in feeding males (figure 2.8), the time at that testicular/ vas deferens enlargement (reviewed by Oliver, 1982) and testicular fluid production occurs.

This is not the first chemical factor to be associated with the male genital tract of ixodid ticks. Shepherd *et al.* (1982) identified a 12.5 kD peptide factor in *Ornithodoros moubata* and *Dermacentor variabilis* associated with their accessory glands that was responsible for spermatozoa capacitation. Chemical factors originating in the male genital tract have also been suggested to provide the stimulus for initiating the rapid engorgement phase. However, neither the nature, nor the specific origin of these factors have been determined (reviewed by Oliver, 1989).

Male factor activity appears not to be restricted to the male genital tract (figure 2.9). The synganglia of either fed males or females, and the ovary of mated fed females also contain male factor like activity. Male factor like activity associated with the ovary could be due to a number of sources. Once the spermatophore is deposited into the female, sperm is transported into the ovary via ovarian muscular contractions (Brinton and Oliver, 1971; Oliver, 1974). Any male factor transported into the female ovary along with spermatozoa could be released during homogenization and thus provide an exogenous source of male factor. Secondly the male factor like activity associated with the ovary of mated females could be merely surface-bound male factor from the haemolymph. If so, this binding may be specific, since male factor can easily be separated from spermatozoa by centrifugation (figure 2.6). Whether the ovary could represent a target tissue of male factor has not been determined.

Male factor like activity in the synganglion of males and females may not be male factor itself. It is possible that a common neurotransmitter substance or intracellular second messenger of the synganglion can mimic male factor activity. For example, in *O. moubata*, a high molecular weight sperm-derived protein complex is responsible for stimulating vitellogenesis and oviposition in mated females (Germond and Aeschlimann, 1977; Sahli *et al.*, 1985; Connat *et al.*, 1986). This protein is released by the sperm cell following capacitation, that occurs during the first hours after mating (Germond and Aeschlimann, 1977; Sahli *et al.*, 1985). While investigating the pathways by that this substance acted, Connat *et al.* (1986) found that implantation of brains from mated females caused a significant increase in vitellogenesis. However, this effect was not specific, as brains from either virgin females or males could also induce comparable levels of vitellogenesis. Conversely, the synganglion and ovary may represent part of the pathway leading to salivary gland degeneration and by injecting these homogenates into virgin female, we are effectively bypassing the step modulated by male factor. In insect systems, male genital tract secretions also play essential roles in the female's reproductive phase. Borovsky (1985), for example, showed that male accessory gland secretions (MAGS) in the mosquito, *Aedes taeniorhynchus*, were able to effect the release of a corpus cardiacum stimulating factor (CCSF) from the ovary. CCSF in turn stimulates the corpus cardiacum to release an egg development neurosecretory hormone (EDNH; Borovsky, 1982) that in turn stimulates the synthesis and release of ecdysone by the ovary (Hagedorn *et al.*, 1979; Hanaoka and Hagedorn, 1980). Several steps may also be involved in linking mating and salivary gland degeneration in *A. hebraeum*.

Male factor appears to act indirectly on the salivary glands. The salivary glands of mated females below the critical weight, although exposed to male factor, do not degenerate (figure 2.3; Harris and Kaufman, 1984). Also, other experiments suggest that male factor does not trigger the synthesis of ecdysteroid receptors in the female salivary gland; injection of male factor into virgin females above the critical weight, however, does elevate haemolymph ecdysteroid titres (Lomas and Kaufman 1992b; see also chapter 4). Male factor may therefore be one of several steps in a chain of events leading to the release of salivary gland degeneration factor that is thought to be an ecdysteroid. Once the factors from the male gonad and other tissues are further characterized, I should be able to comment further on their identities.

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Chapter 3
Biochemical Nature of Male Factor

Introduction:

Most ixodid ticks mate on the host after the blood meal is initiated but before repletion (Gregson, 1944; Khalil, 1970; Balashov, 1972). Because ticks depend on a chance encounter with a host to acquire their blood meal, and the female is able to feed only once as an adult, ticks have developed several strategies to increase the chances of bringing suitable mates together on an appropriate host. These include the use of pheromones (Berger, 1972; Rechav *et al.*, 1976; Allan *et al.*, 1988; Hamilton and Sonenshine, 1988; Sonenshine, 1991), tactile stimulation (Hess and Vlimant, 1986), and various chemical factors that coordinate feeding and detachment, oocyte development, and ovipositional latent period. Most of these chemical factors are derived directly from the male and are transferred to the female during copulation. For example, Pappas and Oliver (1972) demonstrated the importance of a chemical factor, the biochemical nature of which is not known, that is necessary for the female to enter the rapid engorgement phase of feeding and detach from the host. Shepherd *et al.* (1982) purified a 12.5 kD peptide from the male accessory glands that is necessary to complete sperm capacitation, a process that occurs within the female. In argasid ticks, Germond and Aeschlimann (1977) identified a protein that is responsible for initiating oviposition. Later, this protein factor was found to be a high molecular weight (>1,000 kD) protein complex (Connat *et al.*, 1986) secreted by mature spermatozoa (Sahli *et al.*, 1985). Finally, Harris and Kaufman (1984) described a 'male factor' that is derived from the testicular fluid of males and acts on the female to accelerate salivary gland degeneration (see chapter 2).

Whereas I have determined the effect male factor has on salivary gland degeneration and identified the primary location of male factor, I do not know if our male factor is biochemically similar to previously described mating factors. In this chapter, I examine some of the biochemical properties of male factor and demonstrate that male factor is indeed distinct from previously described mating factors.

Materials and Methods:

Ticks:

All ticks used in the following experiments were reared as described in chapter 2 (Materials and Methods, p. 40 of this thesis). Virgin or mated females used in this study weighed 400-1200 mg (the critical weight for *A. hebraeum* is 250-300 mg). All males used were fed for at least 5 days.

Male Factor Bioassay:

The male factor bioassay was performed as follows: Tissues from donor ticks were prepared as described below and injected into virgin females above the critical weight on day 0 post-removal. Injections were made through the camerosomal fold, situated above the capitulum, using a 100 µl Hamilton syringe equipped with a 30 gauge needle, modified to reduce residual volume (see appendix 3). Ticks were then incubated at 26°C, 95% relative humidity for 4 days, after that time the fluid secretory competence of their salivary glands was determined. Controls consisted of mated and virgin ticks above the critical weight, each injected with 1.2% NaCl (saline). Fluid secretory competence of the salivary glands was determined using the fluid secretory assay of Harris and Kaufman (1984; see also p. 40 of this thesis). High fluid transport rates indicate low male factor activity while low fluid transport rates indicate high male factor activity.

Heat Stability:

The genital tracts of 6-10 fed males were removed in ice cold saline, transferred to a 500 µl ground glass homogenizer (containing 10 µl saline/male genital tract), and homogenized. The tissue extract was then transferred to a 500 µl polystyrene centrifuge tube, sealed, and immersed in boiling water for 5 min. A separate tube containing saline alone was also immersed in boiling water for 5 min and acted as the vehicle control. After boiling, homogenates were vortexed well, centrifuged for 5 min at 8000 x **g** and the supernatant injected into virgin females (2 male genital tract equivalents/injection) as described in the male factor bioassay protocol.

Proteinase-K Sensitivity:

For protein degradation experiments, reproductive tract extracts were incubated at 37°C for 1 hour in 0.01M phosphate buffered saline (0.157 g $NaKPO_4$, 17.53 g NaCl l^{-1} distilled, deionized water, pH=7.3) containing proteinase-K (EC.3.4.21.14; 10-20 units[3] per mg lyophilized powder; Sigma) at an enzyme:substrate ratio of 1:3 (w/w). Protein concentration was determined by the method of Lowry *et al.* (1951). Treated extracts were then centrifuged and injected into virgin females as described above. Controls consisted of injecting male factor or Proteinase K alone, each incubated at 37°C for 1 h prior to injection.

Injection of Prostaglandin E_2:

A stock solution of prostaglandin E_2 (PGE_2; Sigma) was prepared in absolute ethanol (4 μg/μl) and stored at -70°C until use. At the time of injection, the stock solution was diluted 1000-fold in saline to a working concentration of 4 ng PGE_2/μl saline (final EtOH concentration = 0.1%). 25 μl (= 100 ng PGE_2) was injected in virgin females as described above. Vehicle injections consisted of 25 μl of 0.1% EtOH in saline.

Size Exclusion Chromatography:

Bio-Gel P6 (medium; Bio-Rad) and Sephacryl S200 (Bio-Rad) chromatographic beads were swollen overnight in 0.01M phosphate buffered saline (0.157 g $NaKPO_4$, 17.53 g NaCl per l distilled, deionized water, pH=7.3), autoclaved for 20 min to sterilize, and equilibrated at 4°C for 24 h. The size exclusion column consisted of a 1.5 cm (internal diameter) x 100 cm Econo-column (Bio-Rad), poured at 4°C using approximately 2.5 ml of Bio-Gel P6 as coarse gel and approximately 150 ml of Sephacryl S200 as the primary gel. The column was packed at 4°C for 24 h at a flow rate of 0.38 ml/min using phosphate buffer as the eluent. This column has a molecular weight separation range of 20-200 kD.

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[3] One unit will hydrolyze casein to produce colour equivalent to 1.0 μmole (181 μg) of tyrosine per min at 37°C (pH 7.5).

Genital tracts from 12-20 fed males were dissected in phosphate buffer and homogenized as previously mentioned. The homogenate was centrifuged at 8000 x **g**, the supernatant collected, combined with phosphate buffer to a final volume of 500 µl, and applied to the top of the column. Separations were carried out at 4°C at a flow rate of 0.38 ml/min, and 1 ml fractions collected. The absorbance of all fractions was monitored at 280 nm. Fractions representing areas under major absorbance peaks were combined and tested for male factor activity in our male factor bioassay. The combined fractions were transferred to dialysis tubing (3.5 kD molecular weight cutoff; Spectra/Por®3, Spectrum, California), dialyzed against deionized, distilled water (4 changes in 24 hrs), frozen at -70°C, and lyophilized (Freezemobile 6, Virtis Inc., New York). Lyophilized material was reconstituted in saline (25 µl/male genital tract loaded on column) and tested for male factor activity as described above. Molecular weight standards used to calibrate the size exclusion column were obtained from Bio-Rad and were: thyroglobulin (bovine; 670 kD), γ-globulin (bovine; 158 kD), bovine serum albumin (66 kD), ovalbumin (chicken; 44 kD), carbonic anhydrase (29 kD), myoglobin (horse; 17 kD), and cyanocobalamine (1.35 kD).

Statistics:

All data are reported as mean ± SEM (N). Statistical significance was determined by one way analysis of variance (ANOVA), Student's T-test, or Mann-Whitney U nonparametric test (using Statview^{TM} SE+Graphics, Abacus Concepts Inc., California, USA) on a Macintosh computer.

Results:

Heat Stability of Male Factor:

When the homogenates of reproductive tract tissues were boiled before being injected into virgin females above the critical weight, all male factor activity was lost, and the fluid transport rate of salivary glands taken from these virgin females equaled that of the virgin controls (2.76 ± 0.56 mg/gland/10 min, N=12, heat treated vs 2.83 ± 0.34 mg/gland/10 min, N=16, virgin control; $p>0.05$; figure 3.1).

Effect of Proteinase-K Treatment:

Crude extracts of male genital tracts lost all male factor activity after being incubated for 1 h at 37°C in the presence of proteinase-K (figure 3.2). Fluid transport of salivary glands decreased from 2.60 ± 0.21 (N=6) to 2.29 ± 0.22 (N=14) mg/gland/10 min when the virgins were injected with proteinase-K treated extract. This compared to a decrease from 2.60 ± 0.21 (N=6) to 1.12 ± 0.20 (N=9) mg/gland/10 min for virgins injected with untreated extracts.

Effect of Prostaglandin E_2:

Injection of 100 ng PGE_2 caused a significant increase in fluid transport in both mated and virgin females (figure 3.3). Virgin females injected with 100 ng PGE_2 showed a net increase in fluid transport from 4.84 ± 0.31 mg/gland/10 min (N=7) to 6.71 ± 0.49 mg/gland/10 min (N=10). The salivary glands of mated females above the critical weight also showed a net increase in fluid transport from 2.03 ± 0.1 mg/gland/10 min (N=6) to 4.07 ± 0.40 mg/gland/10 min (N=6; figure 3.3).

Apparent Molecular Weight of Male Factor:

Figure 3.4 shows a typical elution absorbance pattern of male genital tract homogenates passed through a Sephacryl S200 size exclusion column (solid trace). By combining fractions under the major absorbance peaks, male factor activity was found to occur within fractions 21-27.

Figure 3.1 Effect of heat treatment on male factor activity. Fluid transport rate of salivary glands taken from virgin females 4 days after injection of 2 male genital tract equivalents either boiled for 5 min or not boiled. Virgin and mated controls received injection veichle only. Mean ± SEM.

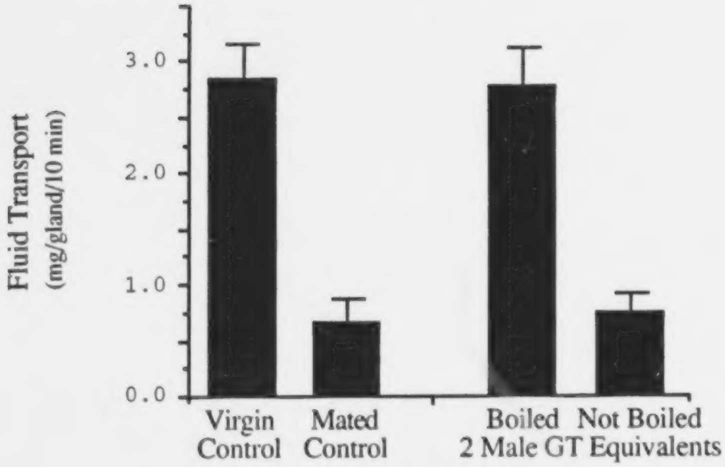

Fluid Transport
(mg/gland/10 min)
3.0
2.0
1.0
0.0
Virgin Control
Mated Control
Boiled
Not Boiled
2 Male GT Equivalents

Figure 3.2 Effect of proteinase-K on male factor activity. Fluid transport rate of salivary glands taken from virgin females above the critical weight 4 days after injection of: genital tract homogenates incubated at 37°C (MF-37°C), genital tract homogenates incubated with proteinase-K at 37°C (MF:Prot-K), or just proteinase-K (Prot-K). Virgin and mated controls recieved only injection veichle. Mean ± SEM.

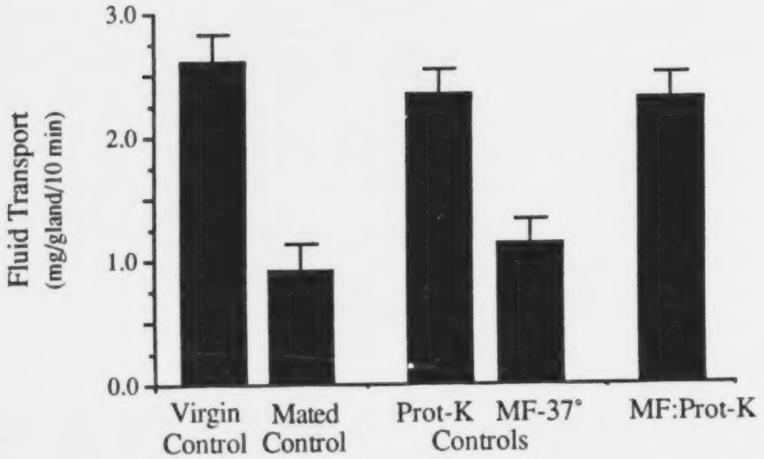
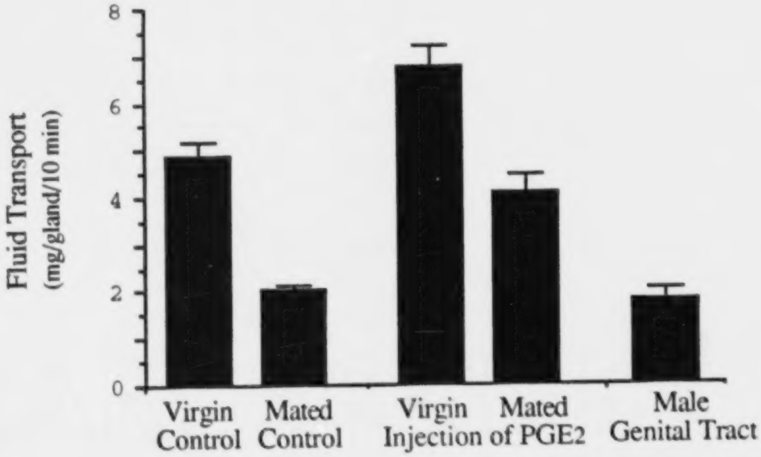
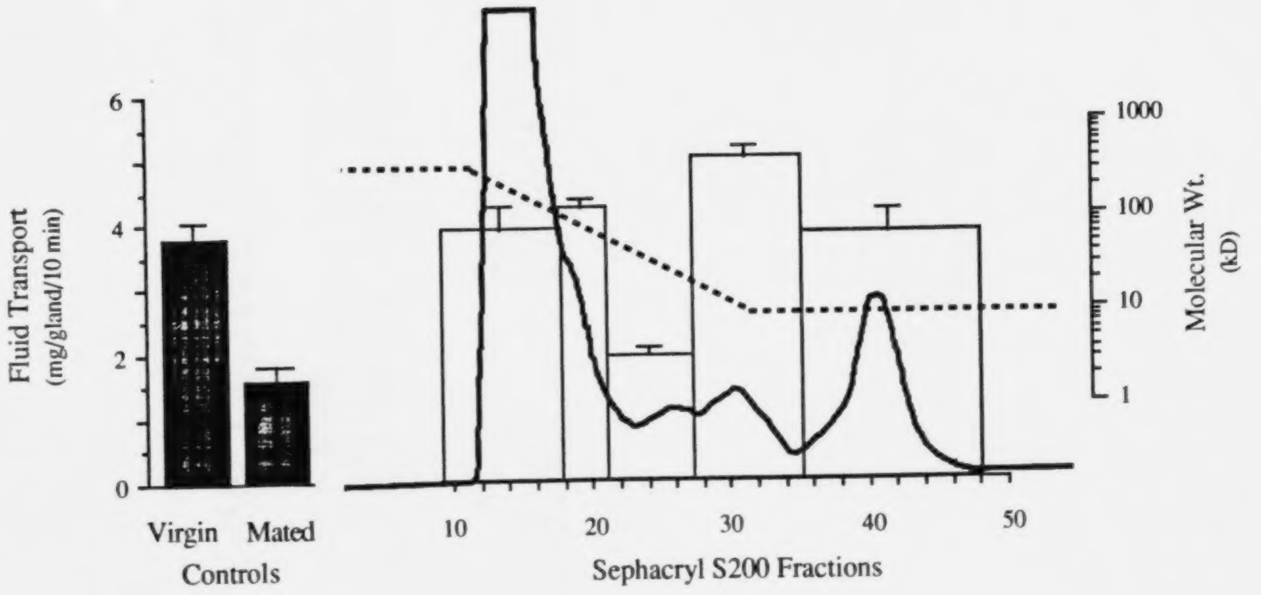

Fluid Transport
(mg/gland/10 min)
3.0
2.0
1.0
0.0
Virgin Control
Mated Control
Prot-K
MF-37°
Controls
MF:Prot-K

Figure 3.3 Effect of prostaglandin E_2 on salivary gland fluid secretory competence. Fluid transport rate of salivary glands taken from virgin or mated females above the critical weight 4 days after injection of 100 ng PGE_2. Controls consisted of injecting virgins and mated females above the critical weight with 1.2% NaCl (containing 0.1% EtOH) or virgins above the critical weight with a male genital tract homogenate (2 male genital tract homogenates/injection). Mean ± SEM.


Fluid Transport
(mg/gland/10 min)
8
6
4
2
0
Virgin Control
Mated Control
Virgin Injection
Mated of PGE2
Male Genital Tract

Injection of this fraction into virgin females above the critical weight caused a reduction in fluid transport from 3.85 ± 0.34 (N=5) to 1.80 ± 0.24 (N=6) mg/gland/10 min. These combined fractions eluted with a K_{av} of 0.19-0.36, that corresponds to an apparent molecular weight of 20-60 kD (dotted line represents the molecular weight calibration curve; figure 3.4).

Figure 3.4 Male factor activity in fractions eluted from a Sephacryl S200 column. Solid trace represents absorbance of each fraction eluted from the column (measured at 280 nm). Dotted line represents the apparent molecular weight calibration curve. White bars represent fluid transport of salivary glands from virgin females above the critical weight 4 days after injection with eluted fractions. Black bars represent controls, either virgin or mated females above the critical weight and injected with 1.2% NaCl. Mean ± SEM.


Fluid Transport
(mg/gland/10 min)
6
4
2
0
Virgin
Mated
Controls
10
20
30
40
50
Sephacryl S200 Fractions
1000
100
10
1
Molecular Wt.
(kD)

Discussion:

Like other male derived factors influencing reproduction in ticks (Germond and Aeschlimann, 1977; Shepherd *et al.*, 1982), male factor is a protein. However, for the following indirect reasons, I suggest that male factor is different from the previously described tick mating factors: (1) I have shown that male factor is primarily located within the testicular fluid and has an apparent molecular weight of 20-60 kD, differing from the protein factor of Shepherd *et al.* (1982) that is derived from the accessory glands and has an apparent molecular weight of 12.5 kD. (2) The mating factor of *O. moubata* (Germond and Aeschlimann, 1977) is of very high molecular weight (> 1,000 kD) and is able to initiate oviposition within 4-5 days of injection. In the absence of this factor, the female can remain in her pre-ovipositional latency period for several months. In our system the absence of male factor does not induce a lengthening of the pre-ovipositional latency (see chapter 2) and the females are able to lay eggs as long as they have fed to an appropriate size and are removed from the host. It would be interesting to test directly the effect of male factor on sperm maturation in ixodid ticks, or the development of oviposition in *O. moubata*.

At this time, it is not known if male factor is distinct from the feeding factor described by Pappas and Oliver (1972) as no biochemical examination of their chemical stimulus has been reported.

Mating factors have been best studied in insect systems and several factors have now been isolated that affect behavioural (Loher *et al.*, 1981; Loher, 1984; Chen and Balmer, 1989a; Chen and Balmer, 1989b; Klowden, 1990) and physiological (Lea and Handel, 1982; Borovsky, 1985; Chen and Balmer, 1989a; Yeh and Klowden, 1990) responses. These include both proteins and prostaglandins. Although the protein nature and molecular weight of male factor excludes it from being a prostaglandin, it is now apparent that in several insect species, a prostaglandin synthetase complex can also be transferred to the female during mating (reviewed by (Stanley-Samuelson and Loher, 1986). PGE_2, prostaglandin $F_{2\alpha}$, ($PGF_{2\alpha}$) and prostaglandin synthetase activity have been reported in saliva, salivary glands, and the reproductive organs of ticks (Higgs *et al.*, 1976; Shemesh *et al.*, 1979; Ribeiro *et al.*, 1992), and the content of PGE_2 and $PGF_{2\alpha}$ increases significantly in both tissues after engorgement (Shemesh *et al.*, 1979). I therefore wanted to test if male factor may be a prostaglandin synthetase complex that acts by increasing PGE_2 titres. Figure 3.3 shows, however, that injection of PGE_2 into virgins above the critical weight did not induce salivary gland degeneration by day 4 post-removal. Instead, PGE_2 caused a significant increase in secretory competence of the salivary glands in virgin and mated females above the critical weight. It thus seems unlikely that male factor exerts its effects through a PGE_2 system.

Further purification of male factor, pending the development of a more sensitive male factor bioassay, is now essential.

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Chapter 4

Mechanism by Which Male Factor Hastens Salivary Gland Degeneration[4]

Introduction:

During the 7-10 day feeding period of female ixodid ticks (Acari: Ixodidae), the blood meal is concentrated in the gut, and the excess fluid is secreted back into the host by way of the salivary glands (Kaufman, 1983). Following repletion, the salivary glands degenerate within 4 days (Harris and Kaufman, 1984). Degeneration is a hormonally controlled process, mediated by the release of 'tick salivary gland degeneration factor' (TSGDF; Harris and Kaufman, 1981). TSGDF is probably an ecdysteroid (Harris and Kaufman, 1985; Lindsay and Kaufman, 1988). Release of TSGDF occurs at a critical weight of approximately 10 times the unfed tick weight and is likely triggered by abdominal stretch (Harris and Kaufman, 1984; Lindsay and Kaufman, 1988). In *Amblyomma hebraeum*, the critical weight is about 300-400 mg.

Mating is an important prerequisite for normal engorgement and reproduction of the female, providing several factors that allow the completion of the blood meal (Pappas and Oliver, 1971; Pappas and Oliver, 1972), initiation of vitellogenesis and oviposition (Connat *et al.*, 1986; Oliver, 1986). If a female remains virgin, she usually will not feed beyond the critical weight and the feeding cycle can be extended to as long as 45 days (see chapter 2).

Mating also provides a 'male factor' (Harris and Kaufman, 1984) that hastens the onset of salivary gland degeneration in detached females that have exceeded the critical weight (Lomas and Kaufman, 1992a; see also chapter 2). In the few virgins that do attain a size above the critical weight (i.e., are competent to release TSGDF but male factor is absent), salivary gland degeneration is delayed until the 8th day after removal. Injection of male factor into large virgins is able to prevent this delay and salivary gland degeneration is complete within 4 days, like that of mated females (see chapter 2). Previous experiments have indicated that male factor is a protein (apparent molecular weight 20-60 kD; see chapter 3) that is transferred to, and stored in the female's seminal receptacle (Harris and Kaufman, 1984); however, the mechanism by that male factor influences salivary gland degeneration remains unknown. Although I have previously suggested that male factor gains access to the haemolymph and acts as a humoral factor, the specific mechanism remains unknown. In this study, I confirm that male factor indeed acts as a humoral factor and examine the possible mechanisms of action by that male factor affects salivary gland degeneration.

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[4] A version of this chapter (see appendix 4) has been published as Lomas and Kaufman. 1992. Archives of Insect Biochemistry and Physiology 21:169-178.

Materials and Methods:

Ticks:

All ticks used in the following experiments were reared as previously described (see chapter 2, Materials and Methods, p. 40 of this thesis). Virgin or mated females used in this study weighed 400-1200 mg (the critical weight for *A. hebraeum* is 250-300 mg).

Male Factor Bioassay:

The male factor bioassay was performed as follows: Tissues from donor ticks were prepared as described below and injected into virgin females above the critical weight on day 0 post-removal. Injections were made through the camerosomal fold, situated above the capitulum, using a 100 µl Hamilton syringe equipped with a 30 gauge needle, modified to reduce most dead space (see appendix 3). Ticks were then incubated at 26°C, 95% relative humidity for 4 days, after that time the fluid secretory competence of their salivary glands were determined. Controls consisted of mated and virgin ticks above the critical weight, each injected with 1.2% NaCl (saline). Fluid secretory competence of the salivary glands was determined using the fluid secretory assay of Harris and Kaufman (1984; see p. 40 of this thesis). High fluid transport rates indicate low male factor activity while low fluid transport rates indicate high male factor activity.

Injection of Male Reproductive Tracts:

Males, fed for at least 5 days, were glued to a petri dish and the reproductive tracts (= testis/vas deferns/ ejaculatory duct) dissected out in ice cold 1.2% NaCl (saline). 10-20 reproductive tracts were pooled, the tissue was homogenized in ice cold saline (10 µl/reproductive tract) and centrifuged at approximately 8000 x **g** for 5 min. The supernatant (= crude extract) was then injected into virgin females (2 reproductive tract equivalents per female) using a 100 µl Hamilton syringe equipped with a 30 gauge needle.

Injection into the ticks was made through the camerostomal fold. Vehicle-injected controls received 20 µl of saline.

Collection of Tick Haemolymph:

Ticks were washed with distilled water, glued to a Petri dish, and anaesthetized under ice for 15-20 min. An incision was made through the integument along each of the dorso-lateral margins using a razor blade scalpel, and gentle pressure was applied to the dorsal surface. The haemolymph exuding from these wounds was collected in 10 µl volumetric capillary tubes. For radioimmunoassay (RIA) of ecdysteroids, haemolymph was transferred directly into glass distilled methanol (BDH Inc., Toronto) and stored at -12°C. For haemolymph injection experiments, 25 µl haemolymph (pooled when necessary) from donor ticks was immediately injected into each recipient virgin female using a 100 µl syringe equipped with a 30 gauge needle.

Ecdysteroid Radioimmunoassay:

The RIA procedure used was that of Kaufman (1990a). Ecdysteroids were extracted from haemolymph samples in 100% glass distilled methanol, evaporated under vacuum, and stored at -12°C until assayed. At the time of assay, samples were reconstituted in an appropriate volume of 0.05 M borate buffer (containing 50 mg bovine serum albumin/100 ml; pH=8.5) to allow at least three 75 µl replicates, yet maintain a predicted ecdysteroid content within the sensitivity of the RIA (10-500 pg ecdysteroid/replicate). To each replicate was added 90 µl radiolabelled ecdysone (45 Ci/mmol α-[23,24-^{3}H(N)]-ecdysone in borate buffer, approximately 8000 cpm; NET 621, New England Nuclear, Boston, MA.), and 45 µl antibody (final dilution 1:3700 E-22 succinylthyroglobulin in buffer:bovine serum albumin; antibody was the gift of Dr. L. I. Gilbert, University of North Carolina). After incubating for 4 h to overnight, the antibody-bound ligand was separated from free ligand by adding 20 µl of 5% protein A (prepared from *Staphylococcus aureus* by Dr. J. C-AMPbell, University of Alberta, according to the method of Kessler, 1981), centrifuging at 8000 x **g** for 5 min, and aspirating away the supernatant. The pellet was resuspended in 50 µl distilled water, transferred to mini-vials containing 5 ml scintillation cocktail (Scintiverse E; Fisher Scientific, Nepean, Ontario, Canada) and radioactivity was monitored on a scintillation counter. For each RIA, a standard curve was run concurrently, using 20-hydroxyecdysone (20-OHE) as standard (Simes, Milan, Italy). Because the antibody shows cross-reactivity with many side chain modified ecdysteroids (Warren and Gilbert, 1986) all data are expressed as 'pg 20-hydroxyecdysone equivalents/µl' (pg EE/µl).

Organ Culture of Salivary Glands:

The organ culture technique was that of Kaufman (1990b). Ticks were first prepared for culture by surface sterilization, 1 min each in 70% ethanol and 1% thimerosal (Sigma), then transferred to a sterile laminar-flow culture hood. Ticks were glued to a sterile plastic Petri dish, covered in sterile TCM 199 (modified as in appendix 2), and the salivary glands dissected out. Salivary glands were washed 3 times in TCM 199 (the third wash also containing the test concentration of 20-OHE), and transferred onto a prepared agar substrate. The agar substrate consisted of equal volumes of 2% (w/v) low-gelling-temperature agarose (Miles Laboratories, Elkhart, Indiana), millipore filter-sterilized (pore size 0.22 µm) double strength TCM 199, 100 µg/µl gentamicin (Sigma), and 2x the desired 20-OHE concentration to be tested. This gave a final working substrate of single strength TCM 199 containing 1% LGT, 50 µg/µl gentamicin, and 1x the 20-OHE concentration to be tested. Above this agar substrate, a volume of sterile TCM 199 (containing 50 µg/µl gentamicin and the desired 20-OHE concentration) was added to sufficiently cover the transplanted salivary glands (approximately 500 µl).

Cultures were incubated at 26°C for 4 days, at that time the salivary glands were tested for fluid secretory competence as described above.

Statistics:

All data are reported as mean ± SEM (N). Statistical significance was determined by one way analysis of variance (ANOVA) or Mann-Whitney U nonparametric test (using Statview™ SE+Graphics, Abacus Concepts Inc., CA, USA) on a Macintosh computer.

Results:

Male Factor Activity in the Haemolymph:

Figure 4.1 indicates that male factor appears in the haemolymph after copulation. Injection of virgin females with haemolymph from mated females, either above or below the critical weight (1.15 ± 0.20, N=12 and 0.56 ± 0.31, N=7 mg/gland/10 min respectively) caused a significant decrease in fluid secretory competence compared to virgin females that received saline alone (2.80 ± 0.40 mg/gland/10 min, N=13; $P<0.001$). This decreased fluid secretory ability, caused by the injection of haemolymph from mated donors, was not significantly different from saline injected mated controls. Injection of haemolymph from virgin donors above the critical weight did not cause a significant decrease in fluid secretory competence (2.16 ± 0.33 mg/gland/10 min, N=18).

Sensitivity of Salivary Glands to 20-OHE:

With the hypothesis that male factor might act by sensitizing the salivary glands to ecdysteroids, I tested the efficacy of 20-OHE to induce salivary gland degeneration in mated or virgin glands after 4 days of organ culture. The dose response curves obtained for mated and virgin females were virtually identical (figure 4.2). Although the salivary glands from virgin females initially secreted at a higher rate than those of mated females (6.27 ± 0.28, N=6 vs 4.90 ± 0.41, N=15 mg/gland/10 min; $P<0.001$), thereafter, there was no significant difference between them. Exposure to as little as 30 pg 20-OHE/µl induced a significant decrease in fluid secretory ability ($P<0.001$). Maximum response occurred at 100-500 pg 20-OHE/µl. As a further control, a series of nontreated mated females above the critical weight were assayed for fluid secretory competence and haemolymph ecdysteroid titres on day 4 post-removal. The salivary glands from these females secreted at a rate of 1.51 ± 0.14 mg/gland/10 min (N=6) while exposed to final haemolymph ecdysteroid titres of 161 ± 37 pg EE/µl haemolymph (N=3; data

Figure 4.1 Male factor activity in haemolymph of virgin females above the critical weight, mated females above the critical weight, and mated females below the critical weight. Virgin and mated controls were injected with veichle alone. Mean ± SEM.

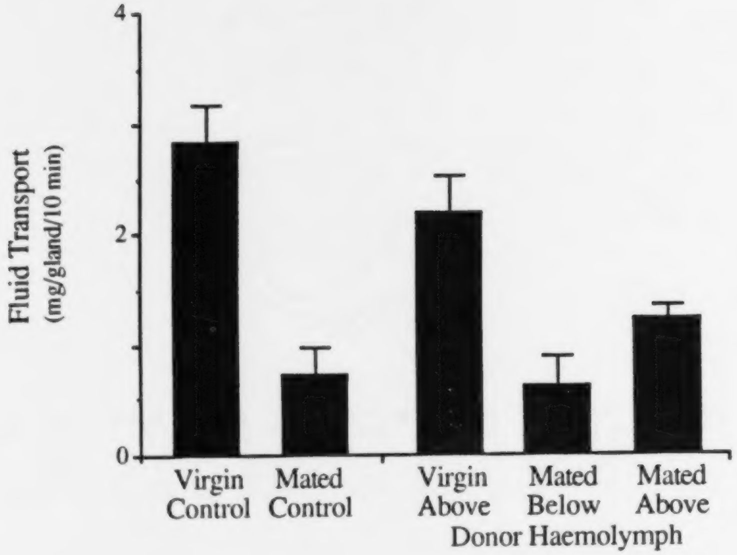
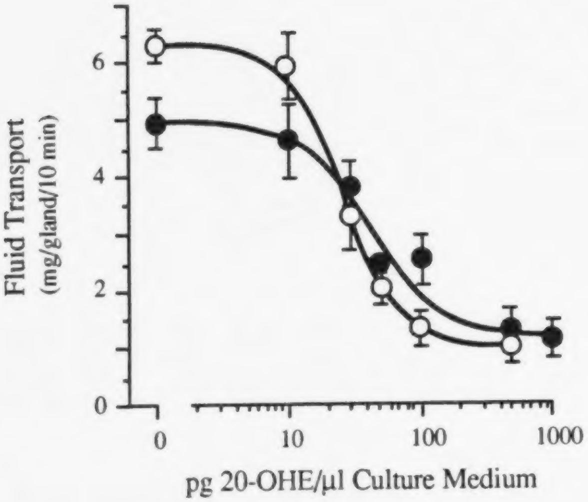

Fluid Transport
(mg/gland/10 min)
4
2
0
Virgin Control
Mated Control
Virgin Above
Mated Below
Mated Above
Donor Haemolymph

Figure 4.2 Fluid secretory competence of salivary glands when cultured in the presence of varying concnetrations of 20-hydroxyecdysone (20-OHE). ○, glands from virgin females above the critical weight; ●, glands from mated females above the critical weight. Mean ± SEM.


Fluid Transport
(mg/gland/10 min)
0
2
4
6
0
10
100
1000
pg 20-OHE/µl Culture Medium

not shown). These figures agree well with the *in vitro* response shown in figure 4.2.

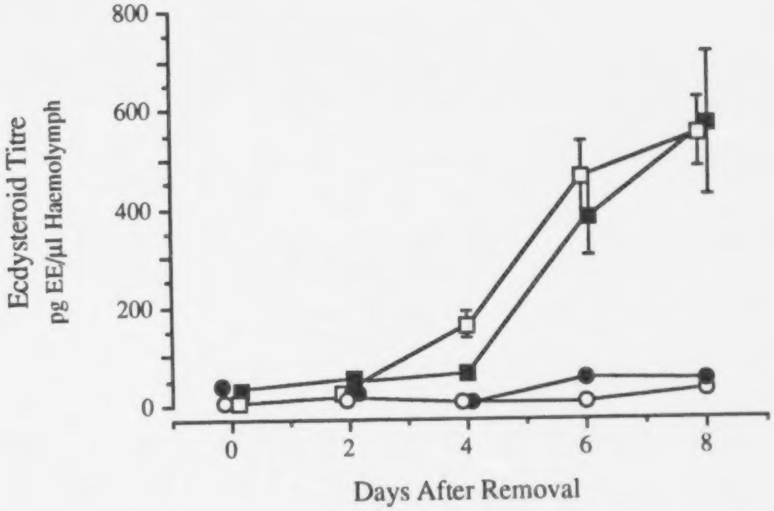
Concentration Profile of Haemolymph Ecdysteroids:

I next measured haemolymph ecdysteroid content of mated and virgin females up to 8 days post-removal to see if there were differences that might account for the delay in salivary gland degeneration in virgins. The ecdysteroid titre of haemolymph from virgin and mated females below the critical weight remained low throughout the 8 day period (<50 pg EE/μl; figure 4.3). The rise in haemolymph ecdysteroid in virgin females above the critical weight was, however, significantly delayed compared to their mated counterparts (figure 4.3). Haemolymph ecdysteroid titres of mated females above the critical weight were initially low (10 ± 1 pg EE/μl, N=6); however, by day 2 they began to increase rapidly and by day 4 post-removal, reached titres of 162 ± 27 pg EE/μl (N=12). Ecdysteroid titres continued to increase, reaching titres of 550 ± 72 pg EE/μl by day 8 (N=8). Conversely, ecdysteroid titres of virgin females on day 0 were significantly higher than that of mated females (30 ± 7 pg EE/μl, N=4; $P<0.001$). The subsequent rise in ecdysteroid titre was, however, attenuated and did not significantly increase by day 4 ($P = 0.12$); the titre in virgins was significantly lower than that of their day 4 mated counterparts (62 ± 9, N=17 vs 162 ± 27, N=12 pg EE/μl respectively; $P<0.001$). Haemolymph ecdysteroid titres began to increase substantially only after day 4, and by day 8 post-removal the haemolymph titre of virgins (567 ± 146 pg EE/μl, N=10) was not significantly different from that of their mated counterparts.

Effect of Male Factor on Haemolymph Ecdysteroid Titre:

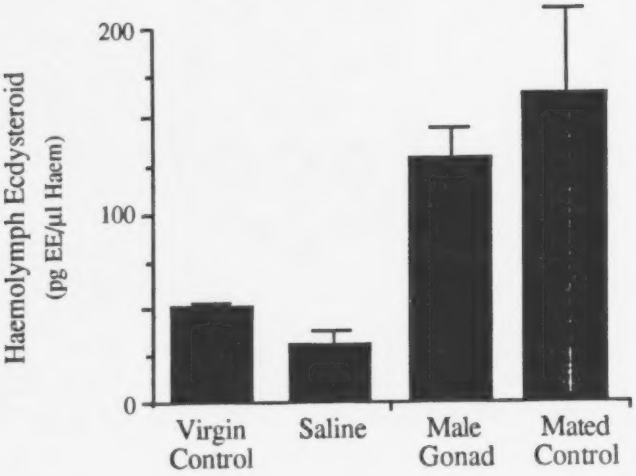
I next tested whether injections of male factor into virgins above the critical weight would hasten the rise of haemolymph ecdysteroid titre. Injection of 2 male

Figure 4.3 Haemolymph ecdysteroid titre measured by RIA as a function of days post-removal from the host, for mated and virgin females above and below the critical weight. □, mated females above the critical weight; ○, mated females below the critical weight; ■, virgin females above the critical weight; ●, virgin females below the critical weight. Mean ± SEM. Where no bar appears, SEM is smaller than the symbol size.


Ecdysteroid Titre
pg EE/µl Haemolymph
800
600
400
200
0
0
2
4
6
8
Days After Removal

reproductive tract equivalents into virgin females above the critical weight caused a significant increase in haemolymph ecdysteroid titres by day 4 as compared to saline injected controls (130 ± 17, N=11 vs 29 ± 7, N=6 pg EE/µl respectively; P<0.001; figure 4.4). This elevated haemolymph ecdysteroid titre was not significantly different from day 4 saline injected mated controls (168 ± 39 pg EE/µl haemolymph, N=7). Imeasured the ecdysteroid content of the injected homogenates to determine if that might account for this significant rise in haemolymph ecdysteroid content. RIA indicated an ecdysteroid content of only 9 ± 6 pg EE/ homogenate injected (N=3). Considering that the haemolymph volume represents about 23% body weight of partially fed ticks and about 10% body weight of engorged ticks (Kaufman *et al.*, 1980), the 9 pg of injected ecdysteroid would have increased the haemolymph titre of a 1000 mg tick by less than 0.1 pg EE/µl.

Figure 4.4 Effect of male factor on the haemolymph ecdysteroid titre. Haemolymph ecdysteroid titres from virgin females above the critical weight was measured 4 days post-removal and injection of either a male reproductive tract homogenate, Male GT, or saline, Saline. Virgin controls were not injected. Mated controls were injected with saline. Mean ± SEM.


Haemolymph Ecdysteroid
(pg EE/µl Haem)
200
100
0
Virgin Control
Saline
Male Gonad
Mated Control

Discussion:

During copulation, the male produces a spermatophore that is then internalized and stored in the seminal receptacle (Moorhouse, 1966). This spermatophore contains sperm, seminal fluid, and male accessory gland secretions. Male factor is probably a component of the testicular fluid (Lomas and Kaufman, 1992a). It has been suggested that male factor leaves the seminal receptacle, enters the haemolymph, and reaches its target tissues(s) as a humoral factor. Harris and Kaufman (1984) ruled out the possibility of male factor acting by a neural mechanism with the observation that severing all nerves from the synganglion to the seminal receptacle did not delay salivary gland degeneration. A humoral mechanism was indicated since surgical removal of the seminal receptacle in mated females and replacement with a male genital tract homogenate (Harris and Kaufman, 1984), or injection of a male reproductive tract homogenate directly into the haemocoel of virgin females mimicked the effect of mating (Lomas and Kaufman, 1992a). Here I show that injection of haemolymph from mated donors directly into the haemocoel of virgin females above the critical weight is also sufficient to initiate salivary gland degeneration (figure 4.1).

I emphasize here that male factor is distinct from TSGDF for at least three reasons. 1) Male factor is abundant in the haemolymph of mated females below the critical weight (figure 4.1), and salivary gland degeneration does not occur in such females for at least 14 days (Harris and Kaufman, 1984). 2) TSGDF is probably an ecdysteroid (Harris and Kaufman, 1985; Lindsay and Kaufman, 1988; Kaufman, 1991). Although the male reproductive tract contains a small amount of ecdysteroid (see above), this exogenous source of ecdysteroid is insufficient to account for the observed salivary gland degeneration. 3) Heat lability, sensitivity to proteinase-K digestion, and gel filtration indicate that male factor is a protein with an apparent molecular weight of

20-60 kD (Lomas and Kaufman 1992a; see also chapter 3).

Evidence presented here indicates that male factor does not act by sensitizing the salivary glands to the action of ecdysteroids (*e.g.*, by up-regulation of ecdysteroid receptors). Thus, salivary glands of virgin females are equally sensitive to 20-OHE as those of mated females (figure 4.2). Instead, male factor appears to act indirectly on the salivary glands by hastening the appearance of ecdysteroids in the haemolymph (figure 4.4). The increase in haemolymph ecdysteroid titre in virgins caused by male factor is sufficient to account for the loss of salivary gland secretory competence in virgin females by day 4. Our organ culture evidence suggests that 30 pg 20-OHE/µl culture medium is sufficient to initiate a reduction in fluid secretory competence, while a complete loss occurs at 100-500 pg 20-OHE/µl. These figures coincide well with the original report by Harris and Kaufman (1985). *In vivo* experiments by Kaufman (1991) correlated natural haemolymph ecdysteroid titres directly with salivary gland secretory competence. He demonstrated a partial reduction in salivary gland function at a mean haemolymph ecdysteroid titre of about 90 pg EE/µl, while degeneration was almost complete at an ecdysteroid titre of 140 pg EE/µl haemolymph; these values are well in agreement with our mated controls of figure 4.2 (161 pg EE/µl). Finally, virgin females above the critical weight show no salivary gland degeneration by day 4; a significant degree of degeneration is apparent only by day 6 (Lomas and Kaufman, 1992a). During this time, the haemolymph ecdysteroid titre rises from 60 pg EE/µl to 379 pg EE/µl (figure 4.3). Considering the fairly large variability inherent in RIA measurements, and that the antibody I used cross reacts with many side chain modified ecdysteroids (Warren and Gilbert, 1986), I feel that the *in vitro* and *in vivo* data are in general agreement. Taking all the foregoing into consideration, the induced rise in haemolymph ecdysteroid titre by male factor injected into large virgins (figure 4.4) does seem sufficient to account for the observed salivary gland degeneration and the proposed mechanism of action. I still do not know how male factor modulates haemolymph ecdysteroid titre. Until an ecdysteroid synthesizing organ is positively identified in adult ticks, the nature of male factor's direct action will remain elusive.

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Chapter 5

Endocrine Source of Ecdysteroids in the Ixodid Tick, *Amblyomma hebraeum*

Introduction:

Ecdysteroids are a family of hormones best known for the role they play in insect development. They stimulate gene specific events that initiate moulting and metamorphosis (reviewed by Riddiford, 1980; Gilbert, 1989), are responsible for the termination of diapause (reviewed by Denlinger, 1985), and stimulate testicular and ovarian development (reviewed by Hagedorn, 1985). Depending on the species and developmental stage, ecdysteroids (3-dehydroecdysone, 3DE, and ecdysone, E) are produced by the prothoracic glands, ring glands, oenocytes, epidermis, and ovary of insects (Bollenbacher *et al.*, 1978) and the Y-organ of crustaceans (Chang and O'Connor, 1977; Spaziani *et al.*, 1989a; Spaziani *et al.*, 1989b). In ticks, ecdysteroids (E and 20-hydroxyecdysone, 20-OHE) were first detected in nymphal *Amblyomma hebraeum* by Delbecque *et al.* (1978). Since that time, a considerable amount of effort has been devoted to the study of tick ecdysteroids; however, the endocrinological source of these hormones, and the physiological mechanisms by that they are regulated are still uncertain. Candidates for the source of ecdysteroids have included the 'lateral segmental organs' (Binnington, 1981), fat body (Ellis and Obenchain, 1984; Schriefer *et al.*, 1987), and more recently, epidermis (Zhu *et al.*, 1991). The latter authors provide convincing evidence that epidermis is the source of E in argasid ticks and although they suggest this is also the case in ixodid ticks, no evidence to support this has yet been presented.

The salivary glands of feeding ixodid ticks are maintained until engorgement, at that time, they degenerate (Harris and Kaufman, 1981; Harris and Kaufman, 1984). Salivary gland degeneration is due to the release of a 'tick salivary gland degeneration factor' (TSGDF; Harris and Kaufman, 1981) that is probably an ecdysteroid (Harris and Kaufman, 1985; Lindsay and Kaufman, 1988; Kaufman, 1991). While examining the effect of mating and 'male factor' on salivary gland degeneration, I have demonstrated that there is a delay in both salivary gland degeneration (Lomas and Kaufman, 1992b; see also chapter 2) and the rise of haemolymph ecdysteroid titres (Lomas and Kaufman, 1992a; see also chapter 4) if the female feeds above the critical weight but remains virgin. Injection of male factor into these virgin females removes both the delay in salivary gland degeneration and rise in haemolymph ecdysteroid titre. On the basis of this indirect evidence, I have suggested that male factor acts primarily by modulating ecdysteroid titres. Direct evidence can only come after identification of the ecdysteroid synthesizing tissue. This chapter describes a series of experiments aimed at identifying the ecdysteroid synthesizing tissues in *A. hebraeum*.

Materials and Methods:

Ticks:

Adult *A. hebraeum* were obtained from a laboratory colony maintained in darkness at 26°C and >95% relative humidity. Adult ticks were confined to the backs of rabbits as previously described by Kaufman and Phillips (1973). For rearing of mated females, equal numbers of males and females were confined together. The rearing of virgin females was modified slightly from that described previously (see chapter 2, p. 40). Males were prevented from mating with females by physically confining them to a porous nylon bag before placing on the rabbit. This bag allowed attachment of males and dissemination of the volatile attachment/aggregation pheromones, but provided a physical barrier to mating. After all females had attached, the bag containing the males was removed.

High-Performance Liquid Chromatography (HPLC):

A Waters Associates HPLC system, comprising a Model 6000A pumping system, Model U6K injector and Series 440 absorbance detector set at 254 nm was used. Ecdysteroids were separated on a C18 μBondapak™ radial compression column (8 mm x 10 cm; Waters Associates) eluted with methanol/water (50:50 v/v) at a flow rate of 1.0 ml/min for 20 min followed with a methanol purge. Samples were collected from the column and assayed for ecdysteroid content using radioimmunoassay (RIA).

Ecdysone Extraction:

Whole Tick Extracts:

Ticks at varying stages of feeding or engorgement were taped to the bottom of a petri dish and cooled for 20 min at 1°C to prevent gut rupture during the initial incision. After cooling, an incision was made around the lateral margin and the dorsum was removed. The gut contents were scraped from the carcass and both placed into a vial containing 3 ml methanol. The sample was vortexed vigorously for 1 min, centrifuged at 14,000 x **g** for 10 min, and the supernatant removed. The remaining pellet was re-extracted twice more in 3 ml methanol, all supernatants combined, and evaporated to dryness under vacuum.

Partial Tick Extracts:

Ticks were cooled for 20 min, as above, flooded with ice cold tissue culture medium 199 (TCM 199, Gibco Inc., MO; modified as described in appendix 2), and a lateral incision made around the entire dorsum. The dorsum was then carefully folded back and away from the body organs, using a razor blade scalpel to sever all tissue attachment. The intact gut was dissected from the tick, ligated with a piece of surgical silk thread (size 000), severed from the esophagus anterior to the synganglion, and transferred to ice cold, TCM 199. It was washed 3 times in fresh TCM 199 and transferred to a vial containing 3 ml methanol. Ecdysteroids were extracted 3 times in methanol, the supernatants combined, and evaporated to dryness as described above. The remaining carcass (including ovary, fat body, synganglion, epidermis, Malphigian tubules, and trachea) was treated, and ecdysteroids extracted in the same way as the gut.

Tick Cultures:

'Backless Tick Cultures':

Ticks were prepared for culture by cooling them under ice (20 min), followed by surface sterilization (1 min each in 70% ethanol and 1% thimerosal; Sigma) in a sterile laminar-flow culture hood. Ticks were glued to a sterile plastic Petri dish (35 x 10 mm), flooded with ice cold filter sterilized TCM 199 (0.22 µm Millex-GV syringe filter unit, Millipore, MA), and a lateral incision made around the entire dorsum. The dorsum was then carefully folded back using a razor blade scalpel to sever all tissue attachment. The body cavity was then flushed twice (2.5 ml each flush) with TCM

199, then a 3rd wash with 2.5 ml of the culture medium. Culture medium consisted of filter sterilized TCM 199 containing (a) 50 µg/ml gentamicin (Sigma), (b) 5×10^{-5} M forskolin (Sigma), and (c) 5×10^{-5} M 3-isobutyl-1-methylxanthine (MIX; Sigma). As much medium as possible was then removed by pipette and 500 µl fresh culture medium added. Cultures were maintained in sterile conditions at 26°C, >95% relative humidity and were considered to be viable during the culture period based on the observation of spontaneous gut and Malphigian tubule contractions as well as by the presence of a leg response following touch. At the time of culture sampling, 100 µl culture medium was removed and assayed for ecdysteroid content by RIA. 100 µl fresh sterile medium was added to the culture following each sampling to maintain constant volumes.

Isolated Tissue Cultures:

Various tick tissues were dissected from engorged females (day 2 post-removal) under sterile conditions (as above), washed 3 times, then maintained in sterile TCM 199 until the time of culturing. Tissues included (a) gut tissue, (b) synganglion, (c) ovary, (d) fat body, and (e) the remaining carcass (containing primarily epidermis, trachea, and muscle tissue). At the time of culture, tissues were transferred to 500 µl culture medium (200 µl for synganglion cultures; medium described above). 100 µl samples (50 µl for synganglion cultures) were removed from each culture at various intervals and ecdysteroid content measured by RIA. An equal volume of fresh culture medium was added back to the cultures after sample removal to maintain a constant volume.

Radioimmunoassay:

The RIA procedure used was that of Kaufman (1990), and has been previously described in this thesis (see chapter 4).

Due to the cross-reactivity of our antibody (gift of Dr. L. I. Gilbert, University of North Carolina, USA) between E, 20-OHE, and many side chain modified ecdysteroids (eg., ecdysonic acid, 26-hydroxyecdysone-22-phosphate; Warren and Gilbert, 1986), all data are expressed as pg 20-OHE equivalents/µl (pg EE/µl).

Statistics:

All data are reported as mean ± SEM (N). Statistical significance was determined by one way analysis of variance (ANOVA) or Mann-Whitney U nonparametric test (using the statistical software package Statview™ 4.0, Abacus Concepts Inc., CA, USA) on a Macintosh computer.

Results:

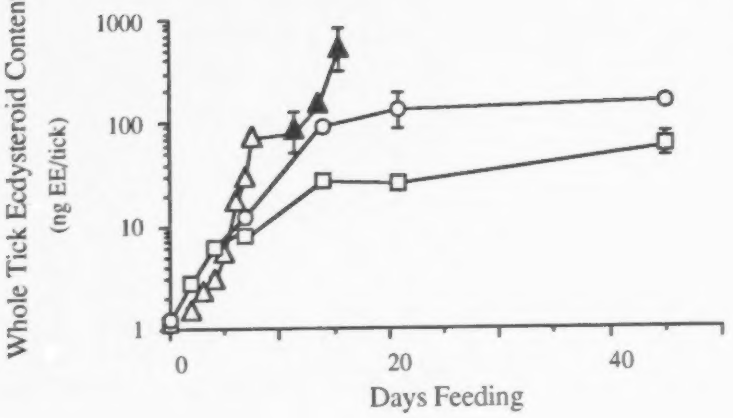
Whole Tick Extracts:

To determine the optimal 'age' of ticks to use as tissue donors for *in vitro* tissue culture experiments, the ecdysteroid content of whole tick extracts was determined on various after the initiation of feeding (figure 5.1). Whole tick extracts from unfed ticks (day 0 feeding) contained 1.3 ± 0.1 ng EE/tick (N=4). This titre increased significantly within 2 days of feeding (2.8 ± 0.3 ng EE/tick; N=4; $p=0.003$), and continued to increase to the end of the period examined (day 8 post-removal, 515 ± 229 ng EE/tick, N=4). In virgin females, ecdysteroid content increased during feeding, paralleling that of mated females, until day 4. Ecdysteroid titres of small virgin females then plateaued at a titre of 28 ± 4 ng EE/tick (N=4) by day 14 and did not significantly change thereafter. Ecdysteroid titres of large virgin females also reached a plateau titre by day 14, however, this plateau (90 ± 9 ng EE/tick, N=4)) was significantly higher than that obtained by small virgin females ($p<0.001$; figure 5.1).

The increase in ecdysteroid content in females that feed beyond the critical weight was highly correlated with feeding ($r^2 = 0.99$, mated and $r^2 = 0.95$, large virgins) and, when normalized for tick weight, mated females maintained a constant ecdysteroid titre of 40-50 ng/g (48 ng EE/g, N=4, unfed vs 43 ng EE/g, N=6, after 7.5 days). Virgin females above the critical weight maintained a slightly higher ecdysteroid content/g tick by day 7 feeding (68 ± 6 ng EE/g, N=4), that slowly increased to a maximum of 206 ± 14 EE/g by 45 days of attachment (N=6). Virgins that remained below the critical weight showed little correlation between ecdysteroid content and tick weight ($r^2 = 0.24$) when feeding periods were extended beyond 21 days.

The apparent discrepancy between the large amount of ecdysteroid in the whole tick by the end of engorgement (figure 5.1) and the small amount found in the haemolymph on

Figure 5.1 Ecdysteroid content of whole tick extracts measured by RIA as a function of days post-attachment for mated and virgin females. △, mated feeding females; ▲, mated engorged females; □, small virgins; ○, large virgins. Data reported as mean ± SEM. Where bars do not appear, SEM is smaller than symbol size.


Whole Tick Ecdysteroid Content
(ng EE/tick)
1000
100
10
1
0
20
40
Days Feeding

day 0 post-engorgement (see chapter 4) suggests that ecdysteroids are selectively partitioned within the tick and these stores are not accessible to the haemolymph. Mated females were therefore dissected, the gut separated from the rest of the carcass, and both portions assayed for ecdysteroid content. As previously shown, more RIA positive material was recovered from ticks above the critical weight (980 ± 116 mg, 67.7 ± 13.5 ng EE/tick, N=4) as compared to ticks below the critical weight (163 ± 45 mg; 14.9 ± 3.0 ng EE/tick, N=4, table 5.1). Of the total amount of recovered ecdysteroid, 78% and 89% was found in the gut of small and large ticks respectively; 22% and 11% (small and large ticks, respectively) was found in the remaining tick carcass (table 5.1). Gut extracts were subjected to HPLC/RIA analysis (see Materials and Methods), and contained large quantities of RIA positive material that co-migrated with authentic E and 20-OHE (figure 5.2). Of the sample injected into the column, 20-OHE and E represented 22% and 55% respectively of the recovered RIA positive material. Thus, E and 20-OHE synthesis appears to begin shortly after attachment and continues until at least the end of the pre-ovipositional latent period.

Tick Cultures:

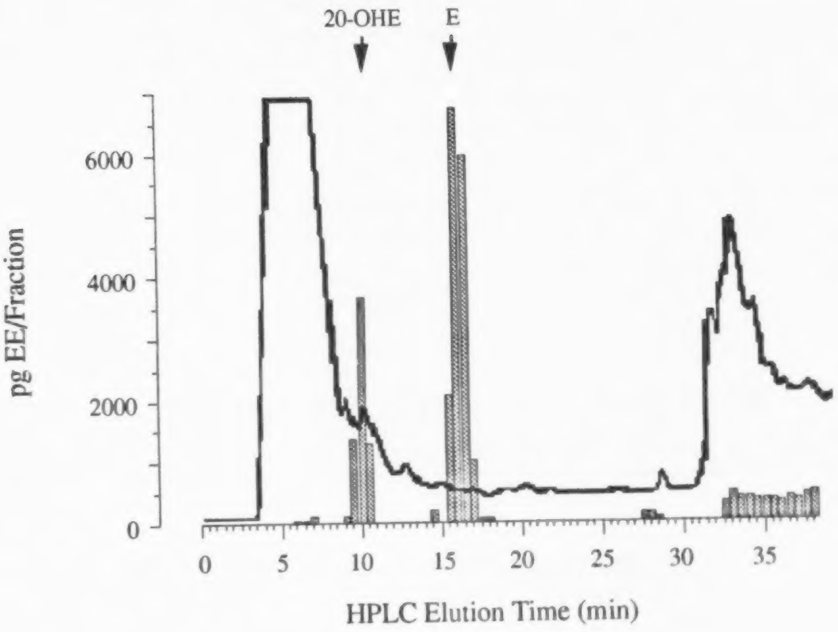
Tissue Culture of Individual Tick Tissues:

Ovary, synganglia, and fat body from mated females, 2 days post-engorgement were maintained in an *in vitro* culture system to assess their ability to synthesize ecdysteroids. Two potent ecdysteroidogenic stimulators in insects, Forskolin (increases c-AMP titres by directly stimulating adenylate cyclase) and 3-isobutyl-1-methylxanthine (MIX; a specific phosphodiesterase inhibitor), were added to all cultures in an attempt to maximize any response. The ecdysteroid content of the culture media from these tissues showed no significant increase when maintained in culture for 24 h (figure 5.3). Cultures of synganglion and ovary showed

Table 5.1 Distribution of ecdysteroids between the gut and remaining carcass (without the haemolymph) Data presented as mean ± SEM.

	Total ng Ecdysteroid	Gut Tissue ng Ecdysteroid	Carcass ng Ecdysteroid
Small Ticks (N=4)	14.9 ± 3.0	11.6 ± 2.1	3.3 ± 1.4
% Total	-	78%	22%
Large Ticks (N=4)	67.7 ± 13.5	60.2 ± 11.5	7.5 ± 2.0
% Total	-	89%	11%

Figure 5.2 HPLC/RIA analysis of gut extract taken from engorged, mated females on day 0 post-removal. Solid trace represents absorption profile of extract (measured at 254 nm). Bars represent RIA positive fractions. Elution of authentic ecdysone (E) and 20-hydroxyecdysone (20-OHE) standards is indicated by arrows.


20-OHE
E
pg EE/Fraction
6000
4000
2000
0
0
5
10
15
20
25
30
35
HPLC Elution Time (min)

a general decrease in culture medium ecdysteroid content from 0 hr to 24 hrs (synganglion: 51 ± 6 to 23 ± 22 pg EE/culture, $p=0.009$; ovary: 33 ± 6 to 14 ± 3 pg EE/culture, $p=0.06$). Cultures of fat body tissue showed a tendency to increase ecdysteroid content (74 ± 9 to 106 ± 21 pg EE/culture) from 0 to 4 h but the effect was not statistically significant ($p=0.17$). Ecdysteroid content then plateaued for the remainder of the experiment (97 ± 15 pg EE/culture by 24 h; figure 5.3).

A long-accepted dogma in insect endocrinology is that the prothoracic glands synthesize E (as a pro-hormone) that is subsequently converted to 20-OHE by the fat body or midgut. Recent evidence has now demonstrated that prothoracic glands of *Manduca sexta* produce 3-dehydroecdysone (3DE; Warren *et al.*, 1988), that is then rapidly converted to E by a 3-ketoreductase enzyme. 3-Ketoreductase is found within the basal laminal sheath that forms the outer boundary of the gland, and in haemolymph (Warren *et al.*, 1988). If tissue cultures were producing 3DE (an E precursor undetectable by our RIA) and if 3-ketoreductase were absent from this system, this might explain the apparent lack of ecdysteroid synthesis in the cultures. I therefore repeated the above tissue culture experiments with the addition of 10 µl haemolymph (taken from day 2 post-removal engorged females) to each culture. Control cultures (no haemolymph added) showed no significant changes in ecdysteroid content (figure 5.4) and behaved as previously described (figure 5.3). The addition of haemolymph to each culture caused an increase in ecdysteroid content, but this was largely due to the endogenous ecdysteroid content of haemolymph (see chapter 4). Culturing synganglion, ovary, or fat body for 24 h in the presence of haemolymph resulted in no significant change in culture medium ecdysteroid content. Conversely, ovary cultures showed a significant decrease in the ecdysteroid content of the culture medium (491 ± 52 to 279± 47 pg EE/culture, $p=0.02$; figure 5.4).

Figure 5.3 Ability of isolated tick tissues to synthesize ecdysteroids *in vitro*. All tissue was removed from donor ticks on day 2 post-engorgement and the ecdysteroid content of culture medium measured 24 h later. □, synganglion; ○, ovary; △, fat body. Data presented as mean ± SEM. Where no bars appear, SEM is smaller than the symbol size.

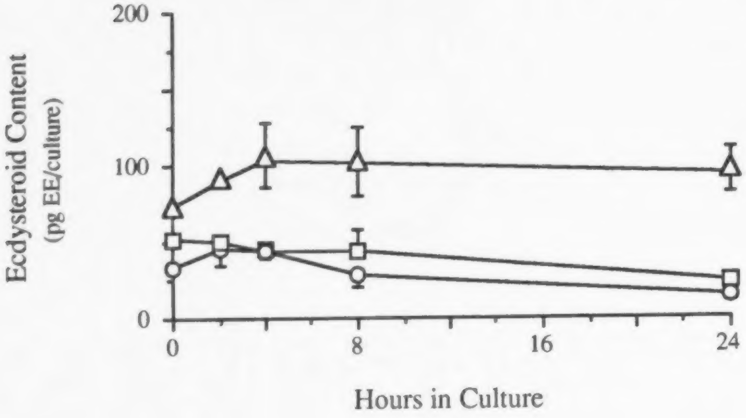
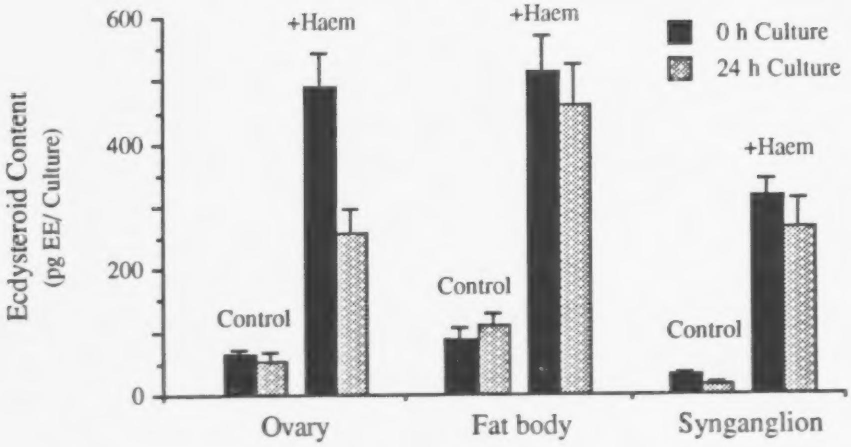

Ecdysteroid Content
(pg EE/culture)
200
100
0
0
8
16
24
Hours in Culture

Figure 5.4 The ability of ovary, synganglion, and fat body tissues to synthesize/release ecdysteroids when maintained in organ culture media supplimented with tick haemolymph. Data reported as mean ± SEM.


Ecdysteroid Content
(pg EE/ Culture)
600
400
200
0
+Haem
+Haem
+Haem
Control
Control
Control
0 h Culture
24 h Culture
Ovary
Fat body
Synganglion

'Backless Tick Cultures':

Because I could not detect direct synthesis of ecdysteroids from isolated tissues, all tick tissues were cultured together in a 'backless tick' preparation (see Materials and Methods). Backless cultures (prepared from engorged females 2 days post-removal) showed a significant increase in culture medium ecdysteroid content during the first 7 h of culture and during the final 52 h of culture (figure 5.5). Ecdysteroid titres increased from 81 ± 7 pg EE/culture (0 h, N=5) to 270 ± 60 pg EE/culture (7 h, p=0.014), then to 1059 ± 214 pg EE/culture ($p<0.0001$) by 100 h after the initiation of the culture.

Given the large quantity of ecdysteroid present in the gut of engorged females (table 5.1), it was important to determine if the increase in ecdysteroid content of the culture medium in backless preparations was due to *de novo* synthesis of ecdysteroid or merely release of RIA positive material from the gut. Sections of gut tissue were cultured individually for 24 h, with or without 0.1M sodium azide as a metabolic inhibitor, and the ecdysteroid content of the culture medium and gut tissue was compared to the content at time 0 h. The total amount of ecdysteroid isolated from each culture, 24 h after initiation, was not significantly different from time 0 culture titres (3873 ± 851 pg EE/ culture, N=5, initial vs 3319 ± 688, N=5, p=0.63, without sodium azide and 2452 ± 249, N=3, p=0.26, with sodium azide; figure 5.6). There was, however, a general shift in ecdysteroids from the gut tissue to the culture medium. After 24 h, the ecdysteroid content of the gut tissue in control cultures tended to decrease (the effect was almost significant; p=0.07) from 3873 ± 854 pg EE/culture to 1904 ± 426 pg EE/culture (N=5) whereas the ecdysteroid content of the culture medium in these cultures significantly increased (from 113 ± 34 to 1415 ± 672 pg EE/culture, p=0.003, figure 5.6). This shift of ecdysteroid was also evident in cultures

Figure 5.5 Ability of a backless tick preparation to synthesize/release ecdysteroids when maintained for up to 120 h. Data presented as mean ± SEM. Where no bars appear, SEM is smaller than the symbol size.

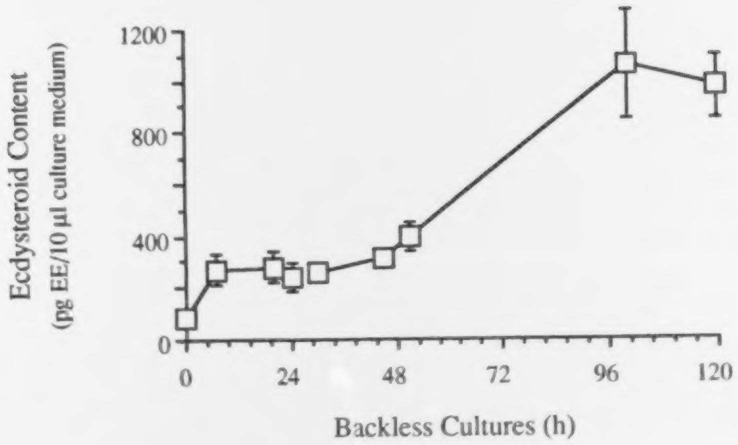
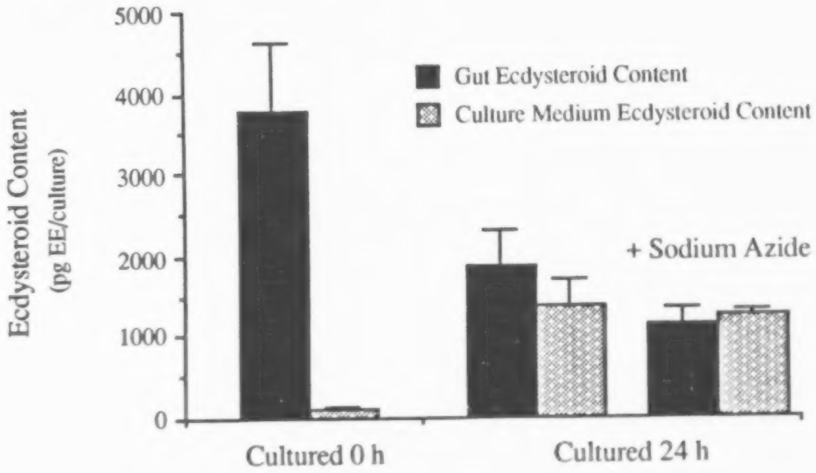

Ecdysteroid Content
(pg EE/10 µl culture medium)
1200
800
400
0
0
24
48
72
96
120
Backless Cultures (h)

Figure 5.6 Passive redistribution of ecdysteroids from gut tissue to the culture medium in an *in vitro* culture system. Data reported as mean ± SEM.


5000
4000
3000
2000
1000
0
Ecdysteroid Content
(pg EE/culture)
Gut Ecdysteroid Content
Culture Medium Ecdysteroid Content
+ Sodium Azide
Cultured 0 h
Cultured 24 h

incubated in the presence of sodium azide, strongly indicating a passive process.

Discussion:

Synthesis[5] of ecdysteroids[6] in female *A. hebraeum* begins shortly after the initiation of feeding, and if they mate, synthesis continues until at least the end of the pre-ovipositional latent period (figure 5.1). These data are in agreement with those of Connat *et al.* (1985), who showed a similar increase in ecdysteroid content in whole tick extracts. The increasing ecdysteroid titre seen in whole extracts of attached females is closely correlated with feeding ($r^2 = 0.99$), and is regulated at a titre of 40-50 ng EE/g. Once the engorged females detach from the host, ecdysteroid titers rise independent of tick weight (43 ng EE/g tick day 0 post-removal vs 318 ng EE/g tick day 8 post-removal) coinciding with the increase in haemolymph ecdysteroid titre (Lomas and Kaufman, 1992; see also chapter 4). This suggests that there may be two phases to ecdysteroid synthesis: an initial phase that is characterized by slow accumulation of ecdysteroid and closely regulated during feeding, and a second phase that begins shortly before engorgement and is characterized by a rapid accumulation of ecdysteroids.

It is unclear why ecdysteroid titres are so closely regulated during feeding. During the initial and slow feeding phases, the epithelial tissue undergoes tremendous enlargement in preparation for the rapid engorgement phase and the subsequent stretch of the cuticle (reviewed by Hackman and Filshie, 1982). Kurtii and Munderloh (1983) demonstrated a dose-dependent effect of 20-OHE on cell attachment rates in two cell lines isolated from *Rhipicephalus appendiculatus* and *Anocentor nitens*; low 20-OHE titres stimulated cell-substrate attachment whereas high 20-OHE titres were inhibitory. In insect epithelial cell lines, 10^{-6} M 20-OHE inhibits cell growth (Fretz *et al.* 1993). Furthermore, high concentrations of exogenously applied ecdysteroids in both ixodid and argasid ticks leads to increased mortality.

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[5] Here I assess synthesis indirectly by an overall change in RIA positive material. It should be realized that the amount of RIA positive material present is due to an equilibrium between synthesis and degradation. Overall changes in RIA positive material reflect changes in one or both of these processes and not necessarily synthesis alone.

[6] Unless otherwise specified, 'ecdysteroids' generically refers to those ecdysteroids that are recognized by our RIA. See Warren and Gilbert, 1986 for a detailed cross-reactivity comparison.

The large accumulation of RIA positive material in feeding and engorged ticks consists largely of both E and 20-OHE (figure 5.2); as much as 22% and 55% of recoverable RIA positive material co-migrating with 20-OHE and E respectively. The majority of these ecdysteroids appear to be associated with the gut tissue, representing 78% and 89% of all recovered material in small and large ticks, respectively. Whether this apparent regionalization is due to a specific sequestering of ecdysteroid in the gut tissue or simply because the gut tissue represents a considerably larger portion of the tick weight is unknown; indirect evidence may suggest it is the former. Kaufman *et al.* (1980) demonstrated that the haemolymph in partially fed (150-600 mg) and engorged females can represent as much as 23% and 10% body weight body, respectively. Given a partially fed female of 500 mg, an engorged (<24 hrs post-engorgement) female of 2 g and an initial ecdysteroid concentration of 10 pg EE/ul haemolymph (Lomas and Kaufman, 1992; see also chapter 4), the total amount of ecdysteroid partitioned within the haemolymph can be calculated to be 1.15 ng EE/small tick and 2.0 ng EE/engorged female. By comparing these values to those obtained by taking 23% of the total ecdysteroid found in partially fed (= 6.67 ng EE, average tick wt = 588 mg) and 10% of the total ecdysteroid found in engorged females (= 7.6 ng EE, average tick wt = 1.78 g), it appears that the haemolymph contains 5.8 and 3.8 times less ecdysteroid (for partially fed and engorged females, respectively) than would be the case if ecdysteroids were distributed equally throughout the body. Although this does not suggest that ecdysteroids are specifically sequestered in gut tissue, it does suggest that pools of ecdysteroids are partitioned within the tick that are inaccessible (or have limited access) to the haemolymph.

Comparison of the ecdysteroid titre of virgin females with mated females indicates that ecdysteroid accumulation is similarly regulated within the first 5 days of feeding. This is expected, as mating does not usually occur until after a few days of feeding (Khalil, 1970). If females do not mate, the rapid accumulation of ecdysteroids seen in mated females does not occur and ecdysteroid content plateaus after 14 days of attachment. Although small virgins plateau at a considerably lower ecdysteroid titre (28 ng EE/tick) as compared to large virgins (90 ng EE/tick), this difference is weight specific. When corrected for tick weight, small and large virgins plateau at similar ecdysteroid titres (125 ng EE/g and 150 ng EE/g for small and large virgins, respectively).

I have previously suggested that male factor acts by modulating the ecdysteroid titre of the haemolymph (Lomas and Kaufman, 1992; see also chapter 4). Present data suggest that this modulation could be achieved during the second, rapid ecdysteroid accumulation phase, or by influencing release of E and 20-OHE from storage tissues. Thus far, it has been impossible to differentiate between these possibilities. My attempts to determine the origin of ecdysteroids in an *in vitro* system have been unsuccessful. The synganglion, ovary, and fat body appear unable to synthesize and/or secrete ecdysteroids, individually or in combination. This could be due to: (1) tissue is developmentally incompetent to secrete ecdysteroids; (2) the culturing conditions do not provide an appropriate stimulatory factor(s); (3) the tissue is not responsible for ecdysteroid synthesis. All tissue used in my culturing system originated from engorged females 2 days post-removal, a time when the ecdysteroid content of both whole extracts (figure 5.1) and haemolymph (Lomas and Kaufman, 1992, see also chapter 4) increases; it is thus unlikely that this lack of synthesis is due to a developmental 'refractoriness'. In insect models, prothoracicotropic hormone (PTTH; immature stages) or egg development neurosecretory hormone (EDNH; adults) are the principle hormones responsible for stimulating ecdysteroidogenesis; both hormones act via c-AMP. Addition of two c-AMP enhancers forskolin and MIX (previously shown to stimulate E synthesis in *Manduca* prothoracic gland cultures; Smith *et al.*, 1989; Shapiro, 1983), had no effect on the ability of tick tissues to synthesize/release ecdysteroids into the culture medium (figure 5.3). It should be noted, however, that ecdysteroidogenesis can also be stimulated through c-AMP independent pathways (Watson *et al.*, 1989).

Although there is a significant release of ecdysteroids into the culture medium by gut tissue, I propose that this is due to a passive release of RIA positive material from the gut into the culture medium because: (1) gut tissue normally contains the major portion of the total E and 20-OHE (table 5.1) and (2) although the ecdysteroid content of culture medium increased, the ecdysteroid content of the entire culture (tissue + medium) remained the same (figure 5.6). (3) Addition of sodium azide to the culture had no effect on the overall ecdysteroid content, nor on the movement of ecdysteroid from the gut tissue to the culture medium (figure 5.6). Results from these experiments are in agreement with those previously found by Zhu *et al.* (1991). They also suggest that gut tissue, synganglia, and fat body are unable to synthesize ecdysteroids *in vitro* (although fat body is able to convert E to 20-OHE). Kaufman (1991) provided evidence suggesting that the ovary is not a source of ecdysteroids by demonstrating that accumulation of ecdysteroids in this tissue occurs only after the haemolymph titre increases. To date, the most convincing evidence suggesting a specific tissue with steroidogenic activity in ticks is given by Zhu *et al.* (1991). They implicate the epidermis as the source of ecdysteroids in the argasid tick, *O. parkeri*. Although they suggest that this is also the case for ixodid ticks, studies with *A. hebraeum* (this study) and *Rhipicephalus appendiculatus* (Dr H. H. Rees, University of Liverpool, personal communication) do not support this.

Since I was not able to demonstrate a steroidogenic tissue within *A. hebraeum*, it is difficult to propose a direct mechanism by that male factor stimulates the rise in haemolymph ecdysteroids. Positive identification of a steroidogenic tissue will be necessary before a specific role for male factor can be ascribed.

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Chapter 6

General Discussion

The life-cycle of most ixodid ticks alternates between a long, fasting, free-living stage and a much shorter parasitic stage; each stage subjects the tick to vastly different physiological pressures concerning availability of water and nutrients. During the free-living stage, female ticks depend on a chance encounter with an appropriate host (a vertebrate that is suitable for parasitism and has fed males attached). Ticks are relatively quiescent during this time and several months (or even years) may pass before a suitable host is encountered (Balashov, 1972). Females have thus adopted a fasting strategy that optimizes their chances of identifying and attaching to a host, while allowing them to survive extended periods without food and water. Adaptations include the ability to absorb atmospheric water to prevent desiccation (Knulle and Rudolph, 1982) and postponement of the development of various digestive and reproductive tissues to conserve nutrient reserves. Conversely, when a suitable host is found and feeding begins, the female changes to a reproductive phase that ensures that the maximum number of eggs isproduced for a given blood meal. Such changes involve development of: 1) the gut epithelium to facilitate digestion (reviewed by Sonenshine, 1991), 2) the salivary glands to maximize fluid secretion (reviewed by Kaufman, 1983), 3) the epidermis to accommodate the enormous volume increase (reviewed by Hackman and Filshie, 1982), 4) the fat body to produce vitellins (Coons *et al.*, 1990), and 5) the gonad itself (Khalil, 1970).

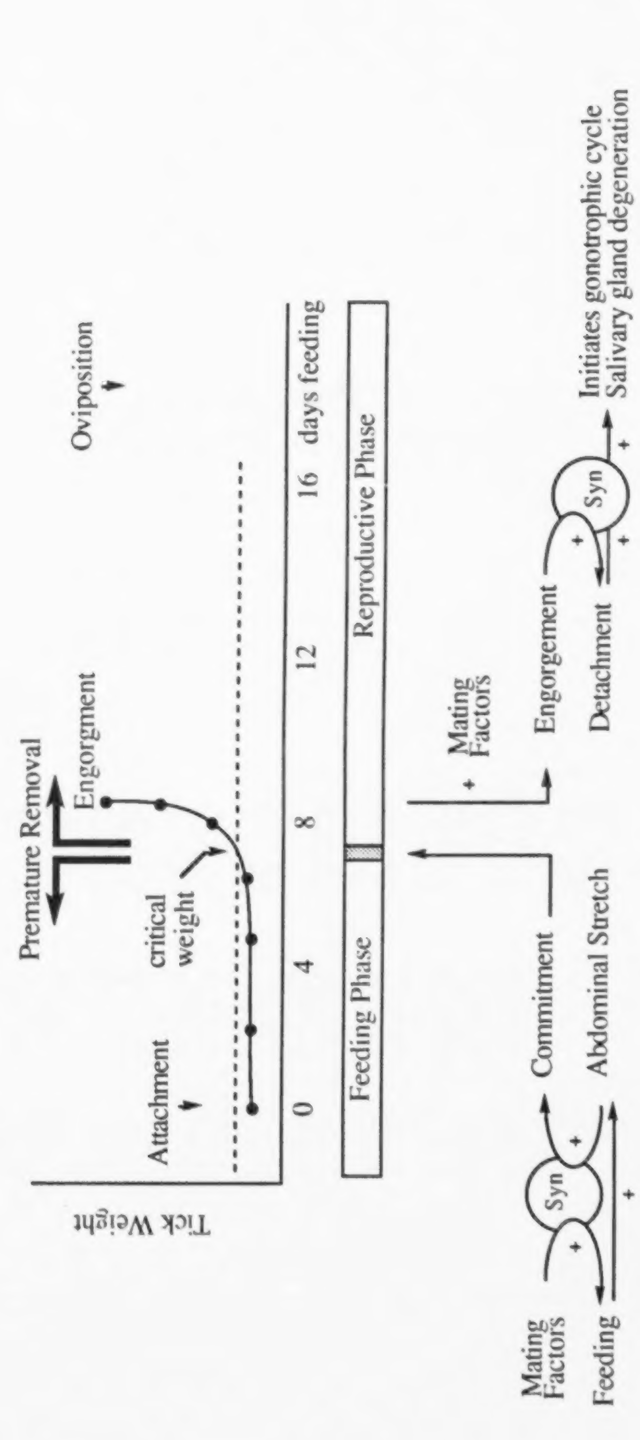
Once a female commits to this reproductive strategy, she apparently becomes incapable to revert to the feeding strategy of water and nutrient conservation, and host location and attachment. Since ixodid ticks are capable of only one gonotrophic cycle, this does not matter if she engorges fully. However, not all females engorge. Ticks can be removed prematurely due to host immunological and/or grooming pressures. Also, if the host should die for any reason, ticks will detach spontaneously. In such situations, the engorgement stage attained at the time of detachment will determine her reproductive success. Presumably, there is some minimum blood meal size for that the benefit of using this nutrient to lay a reduced number of eggs outweighs the risks associated with finding a new host. Indeed, experience in our lab indicates that if forcefully removed females are given the opportunity to reattach, they do so readily only if they are small. As ticks approach the critical weight, females become more reluctant to reattach and once above the critical weight, they will not reattach. Instead, such females will oviposit whatever eggs they can.

The physiological mechanism that initiates the transition from a feeding strategy to a reproductive strategy is not known, but events that seem important are mating and abdominal stretch. Furthermore, once the female has committed to a reproductive strategy, initiation of the gonotrophic phase is delayed until the female detaches. To understand how feeding, mating, and detachment coordinate the successful transition from an unfed to a reproductive strategy, I offer the following model that considers two situations: 1) the female is mated and feeds normally and 2) the female remains virgin.

Feeding of Mated Females (figure 6.1):

Shortly after attachment, the female enters the slow feeding phase where development of gut, salivary gland, epidermis, fat body, and reproductive tissues begin. The female generally mates towards the end of the the slow feeding phase. Rapid engorgement follows and the female acquires the bulk of the nutrients needed for egg laying, with the size of the egg mass produced being directly proportional to the amount of blood taken once the female exceeds the critical weight (Snow, 1969). Unfortunately,

Figure 6.1 Coordination of the 'feeding' and 'reproductive' phase in females that mate. Female *A. hebraeum* typically complete their blood meal within 7-10 days. During this period, the female switches from a feeding phase (retains the ability to locate a new host if necessary) to a reproductive phase (commits to egg production); 'commitment' to the reproductive phase occurs at the critical weight. If females are removed before achieving the critical weight, they retain the ability to locate a new host. If removed after reaching the critical weight, they will instead, oviposit a batch of eggs. I suggest the following mechanism for the involvement of mating factors in determining the females ability to feed to the critical weight, and thus commit to the reproductive phase (see figure 6.2 to contrast). Mating factors stimulate the rate of feeding, that causes increased gut distention. Gut distention is monitored by the synganglion, and once abdominal stretch reaches a critical limit, the female commits to the reproductive phase. Commitment (along with the mating factors) subsequently stimulates rapid engorgement and detachment. Detachment, in turn, provides the 'initiation' stimulus for the gonotrophic phase and salivary gland degeneration.

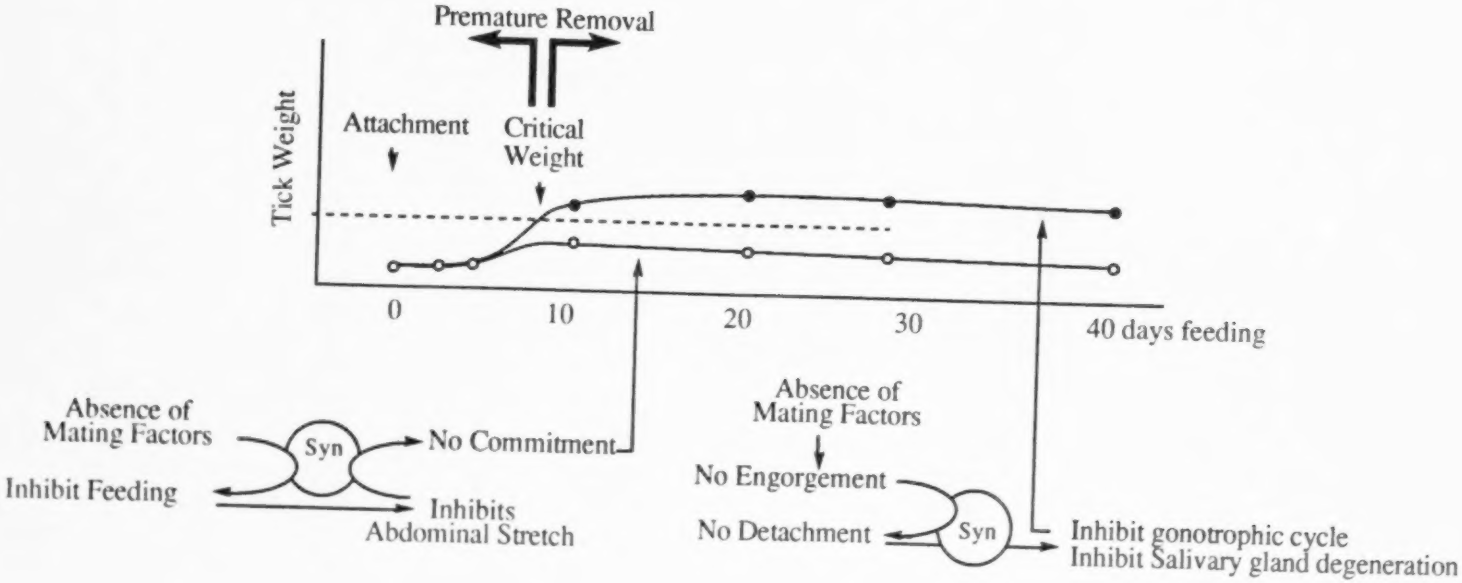

Tick Weight
Attachment
critical weight
Premature Removal
Engorgment
Oviposition
0
4
8
12
16
days feeding
Feeding Phase
Reproductive Phase
Mating Factors
Feeding
Syn
Commitment
Abdominal Stretch
Mating Factors
Engorgement
Detachment
Syn
Initiates gonotrophic cycle
Salivary gland degeneration
+

because of host grooming, etc., some females may be removed prematurely. Such females must then make a choice: look for another host or lay what eggs they can with the small meal. Putative abdominal stretch receptors (Harris and Kaufman, 1984) may be a simple mechanism for making this decision. Ticks below the critical weight (little stretch) cannot lay eggs. Clearly, these females should look for another host. Ticks above the critical weight (more stretch), can lay at least some eggs. Apparently, it is more advantageous to lay even a few eggs than to seek another host.

Feeding of Virgin Females (figure 6.2):

Whereas the situation for mated females seems straightforward, virgins face far more subtle choices. First of all, they also commit to the reproductive strategy once they feed beyond the critical weight (virgins that have fed beyond the critical weight will not reattach). Secondly, they will remain attached to the host (for up to many weeks) waiting for a mate. Thus, they are subjected to greater grooming and host immunological pressures. Finally, with few exceptions, ixodid ticks are not parthenogenic (Oliver, 1971). All this implies that a prematurely removed virgin has no chance for reproductive success if she is above the critical weight. Her best strategy is obvious: if you cannot mate, stay small. This reduces the likelihood of being removed by grooming, but will at least permit her to find another host if she is removed. The above may explain the well-documented reports of most virgins remaining below the critical weight (reviewed by Oliver, 1989; Sonenshine, 1991). Although staying small may be the best strategy, a small number of feeding virgins do obtain a size above the critical weight (see figure 2.1). These females may encounter yet another problem associated with a prolonged stay on a host. The few virgin females that remain on the host for many days in a state above the critical weight would also be subject to premature salivary gland degeneration.

Figure 6.2 The ability of virgins to maximize their reproductive potential in the absence of mating. Ideally, for a virgin to maximize her reproductive success, she should remain below the critical weight; this allows her to find a new host if prematurely detached. In such females, I propose that feeding is inhibited in the absence of mating, thus preventing sufficient abdominal stretch to allow her to commit to a reproductive phase (compare with figure 6.1). In the few virgins that do feed beyond the critical weight, the salivary glands must be maintained until the female mates and engorges (thus the reproductive commitment is separated from initiation). In the absence of mating, I suggest that the inhibition of feeding prevents rapid engorgement, and detachment. Because virgins remain attached to the host, the gonotrophic phase is prevented and salivary gland degeneration does not occur.


Premature Removal
Tick Weight
Attachment
Critical Weight
0
10
20
30
40 days feeding
Absence of Mating Factors
Syn
No Commitment
Inhibit Feeding
Inhibits Abdominal Stretch
Absence of Mating Factors
No Engorgement
No Detachment
Syn
Inhibit gonotrophic cycle
Inhibit Salivary gland degeneration

Since the salivary glands play an essential role in feeding, it would be a poor strategy to finally mate when your salivary glands are no longer functional. Apparently, ticks have solved this potential problem by separating the competence to complete a gonotrophic phase from actually expressing that competence. Some preliminary observations of mine suggest that achieving the critical weight is the 'commitment stimulus' whereas detachment is the 'initiation' stimulus of the gonotrophic phase. Thus, the virgin female seems to have most situations anticipated. If removed below the critical weight, she is little worse off than a similar mated female (both will look for another host). If she exceeds the critical weight, and remains on the host, she commits to reproduce, but will not begin until she mates (thus the salivary glands will remain functional). Being groomed off the host before mating (and above the critical weight) is the only situation where the virgin will not be reproductively successful. I do not know how the female measures her 'attachment state', but one obvious hypothesis involves sensory receptors on the mouthparts.

Mating Factors:

Four mating factors have been isolated to date. At least one of these factors has similar functions in ixodid and argasid ticks, while the others appear unique to each family. Both ixodid and argasid ticks use a 12.5 kD peptide that is responsible for sperm maturation (Shepherd and Oliver, 1982). This peptide, produced by the male accessory gland, mixes with the prospermia at the time of spermatophore transfer and initiates capacitation. Unlike females that attach in the presence of feeding males, male attachment and sperm development is independent of the presence of females. Because considerable time can elapse until a female attaches, a delay in final sperm maturation to the time of copulation may increase sperm survival and thus reproductive success.

Of the three other mating factors identified, two have been identified in ixodid ticks and the third in argasid ticks. The occurrence of distinct mating factors in argasid and ixodid ticks likely reflects fundamental differences in lifestyles. Argasid ticks take substantially less blood over a feeding period that lasts only hours. They can mate off the host, either in a fed or unfed state, and they are capable of several gonotrophic cycles (reviewed by Balashov, 1972). This is in marked contrast to ixodid ticks, that take a single, enormous blood meal over a period of days to weeks, mate on the host (for exceptions, see Oliver, 1989), and are capable of only one gonotrophic cycle. Thus, it may be more relevant here to consider mating factors within ixodid ticks, in that feeding, mating, and reproductive commitment must be more finely tuned. Two such factors have been described: the engorgement factor of Pappas and Oliver (1972) and the male factor of this thesis. Pappas and Olivers engorgement factor directly affects the rate of feeding. When absent, engorgement and commitment to a reproductive strategy are inhibited. Male factor initiates events that allow a more accelerated release of ecdysteroid into the haemolymph when the female is removed (see figure 4.4). I do not yet know if these two factors are distinct proteins.

Future directions:

Several other questions must be addressed before a thorough understanding of the link between mating and salivary gland degeneration can be made. 1) Why do salivary glands degenerate? (A) It seems unlikely that the salivary glands provide a nutrient reserve, since they contain a trivial amount of protein compared to the blood meal (Balashov, 1972). (B) It is possible that the salivary glands provide some essential 'micro-nutrient', however, there is no evidence for this. (C) Salivary gland degeneration may be important to prevent unwanted fluid secretion during egg development. During the gonotrophic phase, ecdysteroid titres are high and may initiate the resorption of substantial amounts of cuticular proteins and lipids. Such resorption could also liberate large amounts of catecholamines (*e.g.*, N-acetyldopamine; fluid secretion is directly stimulated by catecholamines; Kaufman and Phillips, 1973; Kaufman, 1976). Preliminary results by Kaufman and Sloley (unpublished) suggest that haemolymph dopamine titres do not increase by 10 days post-removal; however, the presence of other catecholamines should be sought. (D) Alternatively, salivary gland degeneration may be of no physiological importance; ecdysteroids may have some more important function (see below) and salivary gland degeneration is merely a secondary effect. During larval and nymphal moults, resorption of old tissue and synthesis of replacement tissue allow the salivary glands to grow with increasing body size. Ecdysteroid receptors within the salivary glands of adult females may be a carry-over from the immature stages.

Another important question is: Where are ecdysteroids produced in ticks? Ticks do synthesize ecdysteroids. Unfortunately, the extensive fusion of body segments, the enormous degree to that they feed, and the delicacy of their gut tissue precludes the use of classical ligation-ablation-implantation experiments that were instrumental in determining ecdysteroid sources in other arthropods. Clearly, novel approaches will have to be employed for ticks. One approach that may be successful is the use of radiotracers. Preliminary results from this lab have demonstrated incorporation of radiolabelled cholesterol into 20-OHE in *A. hebraeum*. This technique, although less sensitive than RIA, has the advantage of chemical-physical detection, allowing unambiguous identification of ecdysteroids. With a thorough evaluation of all the ecdysteroids present during the gonotrophic phase, it may then be easier to design experiments that will identify their endocrine source.

Apart from moulting (larval and nymphal stages) and salivary gland degeneration, what physiological roles do ecdysteroids play in adult ticks? In insects, ecdysteroids have definite roles in reproduction. These include: (1) pheromone synthesis (*Musca* domestica); (2) regulation of the corpora allata (*e.g. Nauphoeta cinerea*; (Wilhelm *et al*., 1987); (3) meiotic reinitiation and oocyte maturation (*e.g.*, *Locusta*); (4) control of vitellogenin synthesis (*e.g. Aedes* species); (5) ovulation and uterine contractions (*e.g. Rhodnius* and *Glossina*); (6) incorporation into eggs for use as an embryonic hormone source (*e.g. Schistocerca*). Whether they have similar functions in ticks remains a matter for speculation. Ecdysteroids have been linked to production of the sex pheromone 2,6-dichlorophenol (Dees *et al*. 1984). In males, ecdysteroids stimulate mitosis in spermatocytes, suggesting an important role in sperm development (Oliver, 1992). Induction of vitellin uptake by oocytes has also been suggested, but results from this lab have failed to demonstrate this (Lunke and Kaufman, 1993). They may, however, play a role in fat body development (Lunke and Kaufman, 1993) and vitellin synthesis (Sankhon and Coons, 1992). Incorporation of ecdysteroids into eggs has been demonstrated by several labs; they are assumed to be used by the embryo during the embryonic moults.

Although I still do not know how females successfully coordinate the transition from a feeding strategy to a reproductive strategy, at least we are now acutely aware of these two states and their physiological importance. Once a less tedious male factor bioassay is developed, this *A. hebraeum*-male factor model has great potential to resolve many of these questions.

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Appendix 1

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THE INFLUENCE OF A FACTOR FROM THE MALE GENITAL TRACT ON SALIVARY GLAND DEGENERATION IN THE FEMALE IXODID TICK, *AMBLYOMMA HEBRAEUM*

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Abstract—in the adult female tick, *Amblyomma hebraeum* Koch (Acari: Ixodidae), salivary gland degeneration is triggered by an ecdysteroid, provided the female is above a critical weight (about 300–400 mg). In mated females, salivary gland degeneration is complete within 4 days of detachment from the host. In virgin females, salivary gland degeneration is delayed by 4 days. This delay in salivary gland degeneration could be prevented in virgin females by injecting a homogenate of male gonad into the haemocoel. All "male factor" activity associated with the male gonad resides exclusively within the testis/vas deferens/ejaculatory duct; no male factor activity was associated with the spermatozoa or the accessory gland portion of the male gonad. Male factor activity within the gonad increases markedly after males have fed for 5 days. Male factor can be destroyed by either heat treatment or by proteinase K digestion, suggesting that it is a protein. Some male factor activity was also found in the synganglia of either fed, mated males or females, and the ovary from fed mated females, but not in male salivary glands.

Key Word Index: Ixodid; tick; *Amblyomma hebraeum*; male factor; mating; salivary gland degeneration

INTRODUCTION

Adult female ixodid ticks take an enormous blood meal over their 7–10 day feeding period, often increasing their weight by as much as 100-fold. As feeding progresses, the nutrient portion of the meal is concentrated in the gut through the elimination of excess fluid by salivation back into the host (Kaufman and Phillips, 1973; Tatchell, 1967).

Salivary gland function depends on the degree of engorgement. In unfed *Dermacentor andersoni*, the salivary glands are able to secrete fluid at only a few nl/min (Kaufman, 1976). As feeding progresses their fluid secretory competence increases and eventually reaches a maximum of 200–250 nl/min just prior to engorgement (Kaufman, 1976). Once the female engorges and detaches from the host, the salivary glands degenerate within 3–4 days, a process triggered by the release of a "salivary gland degeneration factor" (Harris and Kaufman, 1981). Release of the degeneration factor is likely triggered by abdominal stretch (Kaufman, 1983) and occurs at a "critical weight" of approx. 10 times the unfed weight (Harris and Kaufman, 1984; Lindsay and Kaufman, 1988). In *Amblyomma hebraeum* the critical weight is about 300–400 mg. Considerable evidence suggests that this degeneration factor is an ecdysteroid (Harris and Kaufman, 1985; Lindsay and Kaufman, 1988); however, so far no ecdysteroid endocrine organ has been identified in ticks.

Mating is an important prerequisite for the normal engorgement of ixodid ticks and degeneration of their salivary glands. Although both the male and female must feed for a few days before mating to allow for genital tract maturation (Khalil, 1970; Feldman-Muhsam and Borut, 1971; Norval and Capitini, 1974), mating usually occurs prior to the female reaching the critical weight. Most females which do not mate will not feed beyond the critical weight and the feeding cycle can be extended to over 40 days (Pappas and Oliver, 1971). Of the few virgin females which do obtain a size above the critical weight, if forcefully removed from the host, their salivary glands do not degenerate within 3–4 days as occurs in their mated counterparts (Kaufman, 1983). Thus, mating affects both feeding and salivary gland degeneration.

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During mating, the male deposits a spermatophore onto the ventrum of the female, which is then internalized and stored in the female's seminal receptacle (Moorhouse, 1966; Feldman-Muhsam *et al.*, 1973). Harris and Kaufman (1984) demonstrated that in *A. hebraeum*, surgical removal of the seminal receptacle from mated females above the critical weight could prevent salivary gland degeneration. Salivary gland degeneration could be restored either by implanting the seminal receptacle into the haemocoel, or by injecting a homogenate of male gonad. Harris and Kaufman (1984) thus demonstrated that in mated females, a male derived chemical factor, which they called male factor, is responsible for promoting salivary gland degeneration within 3–4 days of detachment.

In this study, we look further at the effects of male factor on salivary gland degeneration in virgin female *A. hebraeum*. We also establish the location of male factor within the male gonad and suggest that it is a protein.

MATERIALS AND METHODS

Ticks

Adult *A. hebraeum* were obtained from a laboratory colony maintained in darkness at 26°C and <95% r.h. Adult ticks were confined to the backs of rabbits as previously described by Kaufman and Phillips (1973). For rearing of mated females, equal numbers of males and females were confined together. For rearing of virgin females, males were first treated by plugging their genital pore with a drop of glue (cyanoacrylate compound: Loctite Super Bonder 495, Loctite Corp., Newington, CT, U.S.A.) before confining them with the females. Once all females had attached (12–24 h after placement) all males were removed. Females were later confirmed as virgin by the absence of spermatozoa in a smear of the seminal receptacle. The weights of ticks used in this study are as follows: females below the critical weight = 150–250 mg, females above the critical weight = 400–1200 mg.

Assay for salivary gland secretory competence

The fluid secretory competence of salivary glands was determined according to the method of Harris and Kaufman (1984). Salivary glands were excised from ticks and the main salivary duct ligated with a fine strand unravelled from 8-0 surgical silk thread. The salivary glands were then transferred to tissue culture medium 199 (TCM 199; Gibco Inc., Grand Island, NY, U.S.A.) without sodium bicarbonate, buffered with 10 mM 3-[*N*-morpholino]-propanesulphonic acid (MOPS, Sigma, St Louis, MO, U.S.A.) and osmotically adjusted to 360 mosmol l^{-1} by adding 2.1 gm l^{-1} NaCl. The pH was adjusted to 7.3 using NaOH. At the time of assay, salivary glands were removed from the TCM 199, blotted gently to remove excess medium and the wet weights measured to the nearest 10 μg on a Sartorius 2474 microbalance. Salivary glands were then incubated in constantly stirring TCM 199 containing 10 μM dopamine (Sigma) which elicits a maximal rate of fluid transport (Kaufman, 1976). After a 10 or 15 min incubation period, salivary glands were removed, blotted and reweighed. The net weight increase was taken as an index of fluid secretory competence. All fluid transport rates were normalized to mg gland 10 min.

Male factor bioassay

To test a tissue for male factor activity, the tissue was homogenized in ice-cold 1.2% NaCl, centrifuged and injected into virgin females above the critical weight on day 0 after removal. Virgin ticks were then held at 26°C, >95% r.h. for 4 days, at which time the fluid secretory competence of their salivary glands were determined. Controls consisted of mated and virgin ticks above the critical weight, each injected with 1.2% NaCl. High fluid transport rates indicated a lack of male factor activity while low fluid transport rates indicated the presence of male factor activity.

Injection of tissue homogenates

Tissues from donor ticks were excised and homogenized as described above. The homogenates were then centrifuged at 8000 ***g*** for 5 min and the supernatant (= crude extract) injected into virgin females (either 20 or 25 μl injected volume/female, as indicated) using a 100 μl Hamilton syringe equipped with a 30 gauge needle. Injections were made into the haemocoel through the articulation between the capitulum and the scutum. Controls consisted of injecting an equal volume of 1.2% NaCl. Tissues included: (a) male gonad (either 1 or 2 genital tract equivalents/25 μl) from unfed or fed males, (b) the accessory glands (2 male equivalents/20 μl), (c) reproductive tract with ejaculatory duct (2 male equivalents/20 μl), (d) salivary glands (1 pair/20 μl), (e) ovary from fed, mated females (1 ovary/20 μl), (f) the synganglion from either fed males or females (4 synganglia/20 μl).

Biochemical tests

For heat lability tests, reproductive tract extracts from two fed males were incubated in boiling water for 5 min before centrifugation and injection into virgin females. For protein degradation, reproductive tract extracts were incubated at 37 C for 1 h with Proteinase K (EC 3.4.21.14, Sigma) at an enzyme: substrate ratio of 1:3 (w/w) protein concentration as determined by the method of Lowry *et al.* (1951). Treated extracts were then centrifuged and injected into virgin females as previously described. Controls consisted of injecting male factor or proteinase K alone, each incubated at 37 C for 1 h prior to injection.

Density gradient separation

Reproductive tract homogenates were layered over a discontinuous sucrose gradient consisting of two layers, 1 ml of a 40% sucrose solution (w/w; density = 1.18 gm/ml) and 1.5 ml of a 47% sucrose solution (w/w; density = 1.21 gm/ml). The gradient was centrifuged for 30 min in a Beckman L2-65B ultracentrifuge equipped with a SW 55 Ti rotor at an average of 21,300 *g*. After centrifugation, three fractions could be identified and were labelled top (density < 1.18 gm/ml), middle (density = 1.18–1.21 gm/ml), and bottom (density > 1.21 gm/ml; corresponding to the spermatozoa). Each fraction was removed, washed three times in 1.2% NaCl, injected into virgin females (final concentration = 2 male reproductive tracts/20 μl NaCl) and male factor activity assayed as described above.

Statistics

All data are reported as mean ± SEM (*n*). Statistical significance was determined by one way analysis of variance (ANOVA) using the MIDAS statistical package of the University's main frame computer or Mann–Whitney *U* non-parametric test (using Statview™ SE + Graphics, Abacus Concepts Inc., CA, USA) on a Macintosh computer. Statistical significances are indicated as $*0.05 > P > 0.01$; $**0.01 > P > 0.001$; $***P < 0.001$ and compared to virgin controls unless otherwise indicated.

RESULTS

Time course of salivary gland degeneration

The time course of salivary gland fluid secretory competence in virgin females and mated females above and below the critical weight is shown in Fig. 1. Mated females above the critical weight lost 95% of their fluid secretory ability within 4 days of removal from the host. Mated females below the critical weight lost 71% of their secretory capability within 4 days removal and maintained this secretory level for at least four more days. The time course of fluid secretory competence for virgin females below the critical weight was essentially identical to that of comparably sized mated females. The time course of fluid secretory competence for virgin females above the critical weight was like that of mated females below the critical weight up to day 4 after removal. This level was significantly higher than that of the mated females above the critical weight (2.18 ± 0.24 vs 0.44 ± 0.07 mg/gland/10 min; $P < 0.001$). Secretory competence of the salivary glands from virgin females above the critical weight, however, continued to decline and by day 8 attained a level (0.39 ± 0.07 mg/gland/10 min) not significantly different from that of day 8 mated ticks above the critical weight (0.20 ± 0.06; $P > 0.05$). It thus appears that in virgins above the critical weight, salivary gland degeneration is not completely inhibited as thought earlier (Harris and Kaufman, 1984), but is delayed by 3–4 days.

Male factor activity of various tick tissues

We tested the ability of various tick tissue extracts to hasten salivary gland degeneration in virgin females above the critical weight. Extracts included

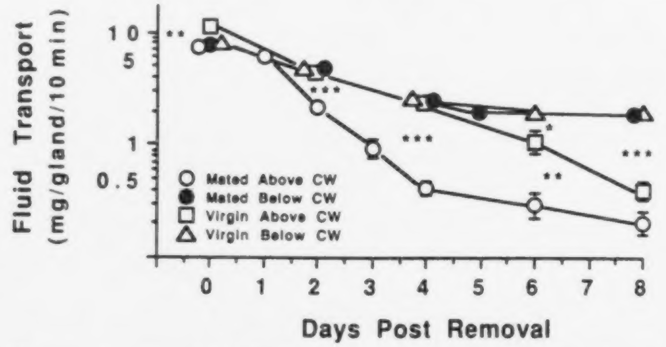

Fig. 1. Fluid secretory competence of salivary glands as a function of time after removal. Mean ± SEM (SEM is shown wherever it exceeds the dimension of the symbol), $n = 7$–42. Statistical significance was determined using the Mann–Whitney *U* test.

the male gonad, male salivary glands, the synganglion of both fed males and females, and the ovary from fed mated females (Fig. 2). Injection of virgin females above the critical weight with a homogenate of male gonad caused a significant, dose-dependent decrease in fluid secretory competence compared to virgin controls. Salivary gland tissue contained no detectable male factor activity. The synganglion from either fed males or females, or the ovary from fed females showed a small but significant amount of male factor activity ($P < 0.01$).

Location of male factor activity within the male gonad

The male gonad is composed of a testis/vas deferens and an accessory gland portion, both of which empty into the ejaculatory duct. Male factor activity occurred within the testis/vas deferens/ejaculatory duct exclusively (Fig. 3). Further localization of male factor within the reproductive tract was achieved by the use of a discontinuous sucrose density gradient (Dawson and Scott, 1964). We confirmed that this technique, originally devised for the separation of pig spermatozoa, could also separate tick spermatozoa (density > 1.21 g/ml) from the seminal fluid and other tissue. Most male factor activity was associated with the middle fraction (density between 1.18–1.21 gm/ml), and some with the top fraction (density < 1.18 gm/ml) but no activity was associated with the spermatozoa fraction (Fig. 3). Thus, male factor is associated primarily with some tissue other than the spermatozoa, or perhaps the seminal fluid itself.

Effect of feeding on male factor activity

As mentioned earlier, before males will mate they must feed for several days to stimulate the final stages of genital tract maturation. Figure 4 shows that feeding markedly increases male factor activity within the gonad.

Biochemical nature of male factor

Male factor is labile to heat treatment (5 min at 100°C) and to proteinase K digestion (1 h at 37°C; Fig. 5). Heat treatment destroyed male factor activity totally. Treatment with proteinase K reduced male factor activity by 58%. These two tests suggest that male factor is a protein.

DISCUSSION

Following the blood meal, the salivary glands of female ixodid ticks degenerate under the influence of a tick salivary gland degeneration factor (Harris and Kaufman, 1981), which is probably an ecdysteroid (Harris and Kaufman, 1985). When identifying some of the components in the pathway leading to the release of this degeneration factor, Harris and Kaufman (1984) implicated a substance originating in the male gonad which they called male factor. At that time, it was thought that male factor might be an essential component in the total pathway. Here we must modify this view with the demonstration that salivary gland degeneration can proceed in the absence of male factor, although the process is delayed by 3–4 days (Fig. 1). With the further

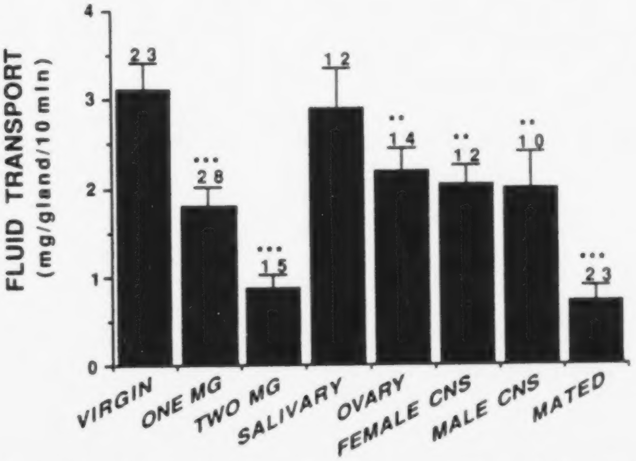

Fig. 2. Male factor activity in various tick tissues: fluid secretory competence of salivary glands taken from virgin females above the critical weight 4 days after injection with either one or two male gonad equivalents (ONE MG or TWO MG), salivary gland (SALIVARY), male or female synganglion (MALE CNS, FEMALE CNS), or ovary from mated females above the critical weight (OVARY). VIRGIN = virgin or MATED = mated controls, each above the critical weight and injected with 25 μl < 1.2% NaCl. Mean ± SE(n). Statistical significance was determined using the Mann–Whitney U test.

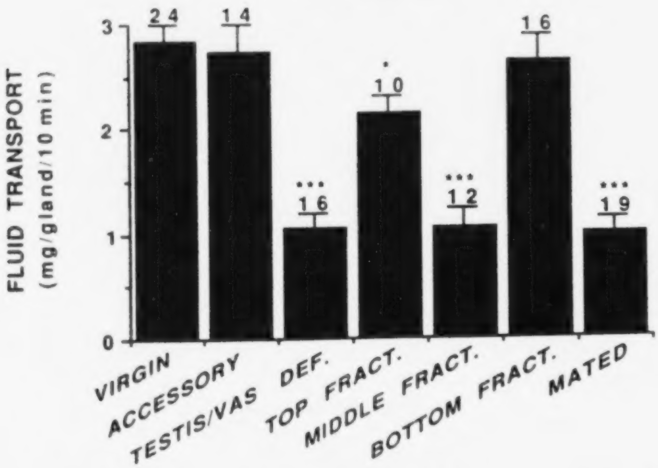

Fig. 3. Localization of male factor activity in the male gonad: fluid secretory competence of salivary glands from virgin females above the critical weight 4 days after injection with either a testis/vas deferens/ejaculatory duct homogenate (TESTIS/VAS DEF.) or an accessory gland homogenate (ACCESSORY). Testis/vas deferens/ejaculatory duct homogenate was further separated into three fractions (TOP FRACT., MIDDLE FRACT., BOTTOM FRACT.) using a discontinuous density gradient centrifugation and tested for male factor activity as above. VIRGIN = virgin or MATED = mated controls, each above the critical weight and injected with 25 μl < 1.2% NaCl. Mean ± SE(n). Statistical significance was determined using the Mann–Whitney *U* test.

evidence that male factor is also able to hasten the release of ecdysteroids into the haemolymph of large virgins (unpublished), it appears that male factor is performing a modulatory rather than essential role. Whatever role male factor plays, it is bound to be subtle, and likely a challenge to uncover.

In this study, we found that a dose of two male gonad equivalents was required to achieve the maximal effect, whereas Harris and Kaufman (1984) found that one male gonad equivalent was sufficient. The discrepancy may be due to the fact that here we used virgins (thus never exposed to male factor) while Harris and Kaufman (1984) worked with mated ticks which had been surgically deprived of the seminal receptacle. It now appears that male factor is normally transferred from the seminal receptacle to the haemolymph of mated females (unpublished). Perhaps these mated, operated females used by Harris and Kaufman (1984) retained some subthreshold amount of male factor within the haemolymph so that a lower exogenous dose was fully effective.

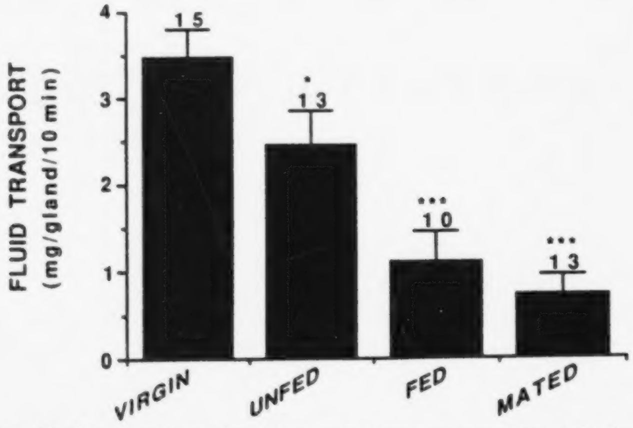

Fig. 4. Effect of feeding on potency of male factor: fluid secretory competence of salivary glands taken from virgin females above the critical weight 4 days after injection with genital tract homogenates from unfed males (UNFED), or males fed for 5 days (FED). VIRGIN = virgin females or MATED = mated controls, each above the critical weight and injected with 25 μl NaCl. Mean ± SE(n). Statistical significance was determined using one way ANOVA.

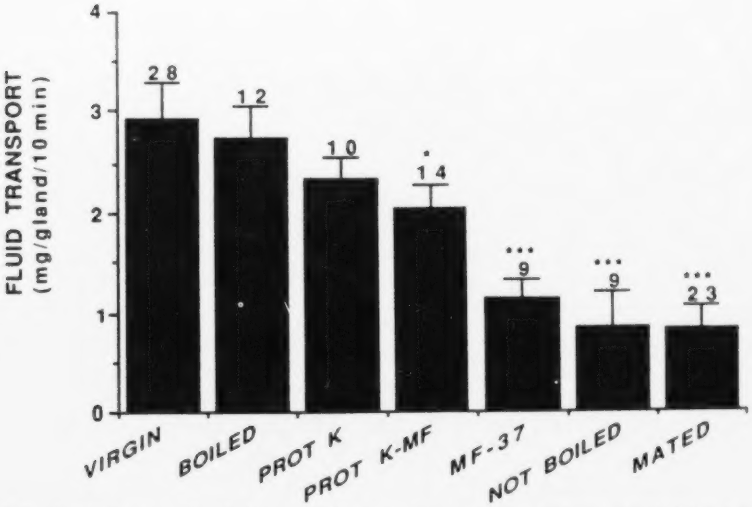

Fig. 5. Biochemical nature of male factor: fluid secretory competence of salivary glands taken from virgin females above the critical weight 4 days after injection of male gonads subjected to either heat treatment (BOILED vs NOT BOILED) or proteinase K digestion at 37°C (PROT K-MF vs proteinase K alone, PROT K, or male factor alone, MF-37). VIRGIN = virgin or MATED = mated controls, each above the critical weight and injected with 25 μl < 1.2% NaCl. Mean ± SE(*n*). Statistical significance was determined using the Mann–Whitney *U* test.

Within the male gonad, male factor appears to be primarily localized within the testis/vas deferens, and more specifically, it is presumably associated with the seminal fluid. Our evidence for this, although indirect, is that: (1) male factor is not associated with the spermatozoa as it can be easily separated by density gradient centrifugation (Fig. 3), and (2) male factor activity is markedly increased in feeding males (Fig. 4), the time at which testicular/vas deferens enlagement (reviewed by Oliver, 1982) and seminal fluid production occurs.

This is not the first chemical factor to be associated with the male gonad of ixodid ticks. Shepherd *et al.* (1982) identified a 12.5 kDa peptide factor in *Ornithodorus moubata* and *Dermacentor variabilis* associated with their accessory glands which was responsible for capacitation of spermatozoa. Chemical factors originating in the male gonad have also been suggested to provide the appropriate stimulus for initiating the rapid engorgement phase. However, neither the nature, nor the specific origin of these factors have been determined (reviewed by Oliver, 1989).

Male factor activity appears not to be restricted to the male gonad (Fig. 2). The synganglia of either fed males or females, and the ovary of mated fed females also contains male factor activity. This may, however, not represent a physiological source of male factor. Once the spermatophore is deposited into the female, sperm is transported into the ovary via ovarian muscular contractions (Brinton and Oliver, 1971; Oliver, 1974). Any male factor transported into the female ovary along with spermatozoa could be released during homogenization and thus provide an exogenous source of male factor. Male factor activity of the ovary and synganglion may also represent other steps in a complex pathway leading to male factor activity, such as a common second messenger or a factor still unknown.

Male factor appears to act indirectly on the salivary glands. The salivary glands of mated females below the critical weight, although exposed to male factor, do not degenerate [Fig. 1 and Harris and Kaufman (1984)]. Also, other experiments suggest that male factor does not trigger the synthesis of ecdysteroid receptors in the female salivary gland (unpublished). Injection of male factor into virgin females above the critical weight, however, does elevate haemolymph ecdysteroid levels (unpublished). Male factor may therefore be one of several steps in a chain of events leading to the release of salivary gland degeneration factor. Once the factor from male gonads is further characterized, we should be able to determine whether this activity is due to the same substance as those found in female ovary and the synganglion of both sexes.

The chemical nature of male factor is largely unknown. The two basic tests conducted here suggest that it is a protein (Fig. 5). In insects, male genital tract factors important in regulating physiological processes within the female have been primarily categorized as peptide factors and prostaglandin factors. One such prostaglandin factor is the prostaglandin synthetase complex. In ticks, prostaglandins (prostaglandin E_2 and prostaglandin F) and prostaglandin synthetase activity has been reported in both the salivary glands and reproductive organs of *Hyalomma anatolicum excavatum* (Shemesh *et al.*, 1979) and the content of prostaglandin E_2 and prostaglandin F in both tissues increases significantly after engorgement. Whether male factor represents a protein factor or the prostaglandin synthetase complex has yet to be determined.

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Appendix 2

Composition and Modification of Tissue Culture Medium 199:

The composition of tissue culture medium 199 (TCM 199) without sodium bicarbonate (Gibco Inc., Grand Island, New York) is shown below. TCM 199 was buffered with 10 mM 3-[N-morpholino]-propanesulphonic acid (MOPS; Sigma, St Louis, Mo., U.S.A.) and osmotically adjusted to 360 mosmol l^{-1} by adding 2.1 gm l^{-1} NaCl. The pH was adjusted to 7.26 using 0.1N NaOH.

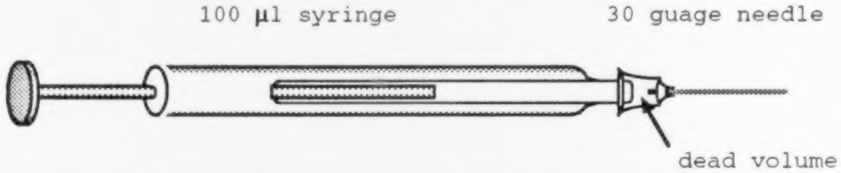
Compostion of Tissue Culture Medium 199

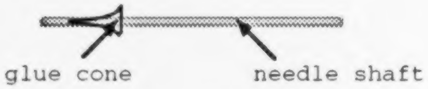
Chemical	mg/L	Chemical	mg/L
$CaCl_2$	140	Ascorbic Acid	0.05
$Fe(NO_3)_3 \cdot 9H_2O$	0.72	α-Tocopherol Phosphate	0.01
KCl	400	d-Biotin	0.01
KH_2PO_4	60	Calciferol	0.1
$MgSO_4 \cdot 7H_2O$	200	D-Ca Pantothernate	0.01
NaCl	8000	Choline Chloride	0.5
Na_2HPO_4 (anhyd.)	47.7	Folic Acid	0.01
DL-Alanine	50	i-Inositol	0.05
L-Arginine HCl	70	Menadione	0.01
DL-Aspartic Acid	60	Niacin	0.025
L-Cystine $HCl \cdot H_2O$	0.11	Niacinamide	0.25
L-Cystine 2HCl	26	Para-aminobenzoic Acid	0.05
DL-Glutamine	150	Pyridoxal HCl	0.025
L-Glutamine	100	Pyridoxine HCl	0.025
Glycine	50	Riboflavin	0.01
L-Histidine $HCl \cdot H_20$	21.88	Thiamine HCl	0.01
L-Hydroxyproline	10	Vitamine A (acetate)	0.14
DL-Isoleucine	40	Cholesterol	0.2
L-Lysine HCl	70	Deoxyribose	0.5
DL-Methionine	30	D-Glucose	1000
DL-Phenylalanine	50	Glutathione (reduced)	0.05
L-Proline	40	Guanine HCl	0.3
DL-Serine	50	Hypoxanthine (Na Salt)	0.354
DL-Threonine	60	Phenol Red	20
DL-Tryptophan	20	Ribose	0.5
L-Tyrosine	57.66	Sodium Acetate	50
DL-Valine	50	Thymine	0.3
Adenine Sulfate	10	Tween 80	20
ATP	1.0	Uracil	0.3
Adenylic Acid	0.2	Xanthine (Na Salt)	0.344

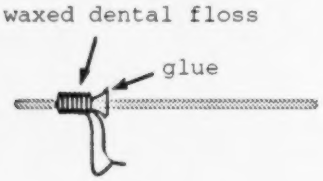
Appendix 3

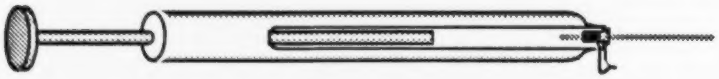
Modification of a 30-guage needle to reduce dead volume:

The 'dead space' of a typical syringe needle is 20-30 µl. Given the small sample volumes prepared and injected into ticks for this thesis, 30-gauge needles were modified to reduce this dead space to less than 5 µl. The modification is as follows: A one sided razor blade was used to cut away the plastic housing surrounding the needle shaft. This exposed a small cement cone (used to hold the needle shaft in the plastic housing) that has a diameter slightly smaller than the bore of a 100 µl Hamilton syringe. This cement cone was wrapped in a waxed Teflon thread (such as dental floss) so that the needle shaft fit snugly within the bore of the syringe. The syringe, with needle inserted, was then autoclaved for 5 min (to melt the wax of the thread), then cooled (seals the tip).









heat to remelt wax and seal tip

Appendix 4

Archives of Insect Biochemistry and Physiology 21:169–178 (1992)

An Indirect Mechanism by Which a Protein From the Male Gonad Hastens Salivary Gland Degeneration in the Female Ixodid Tick, *Amblyomma hebraeum*

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In the adult female tick, *Amblyomma hebraeum* Koch (Acari: Ixodidae), salivary gland degeneration is triggered by an ecdysteroid, provided the female is above a critical weight (approximately 300–400 mg). In mated females, salivary gland degeneration is virtually complete within 4 days of detachment from the host. In virgin females, salivary gland degeneration is delayed by 4 days. This delay can be reversed by the injection of a male reproductive tract homogenate directly into the hemocoel. In this study, we consider a possible mechanism of action for this "male factor." Once mated, male factor likely gains access to its target tissue(s) as a humoral factor. Male factor, however, appears not to act by sensitizing the salivary glands to the action of ecdysteroids. Instead, it appears to act by accelerating the appearance of ecdysteroids in the hemolymph.

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Key words: reproduction, ecdysteroids, male factor

INTRODUCTION

During the 7–10 day feeding period of female ixodid ticks (Acari: Ixodidae), the blood meal is concentrated in the gut, and the excess fluid is secreted back into the host by way of the salivary glands [1]. Salivary gland function is dependent on the degree of engorgement. Salivary glands of unfed adults are virtually incompetent to secrete fluid. As feeding progresses, the salivary glands show a dramatic increase in fluid secretory competence and reach a

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maximal level just before engorgement [2]. Following repletion, the salivary glands degenerate within 4 days. Degeneration is a hormonally controlled process, mediated by the release of TSGDF* [3]. TSGDF is probably an ecdysteroid [4]. Release of TSGDF occurs at a critical weight of approximately 10-fold the unfed tick weight and is likely triggered by abdominal stretching [5,6]. In *Amblyomma hebraeum*, the critical weight is about 300–400 mg.

Mating is an important prerequisite for normal engorgement of the female, and occurs on the host during feeding. If a female remains virgin, she usually does not feed beyond the critical weight and the feeding cycle can be extended to as much as 40 days [7,8]. Of the few virgins that do attain a size above the critical weight, salivary gland degeneration is delayed until the 8th day postremoval; this 4 day delay is due to the absence of a specific protein ("male factor") which is transferred to the female during copulation [5,9]. We have suggested that male factor gains access to the hemolymph and, by a mechanism yet unknown, accelerates salivary gland degeneration [9].

In this study, we show that once the female has mated, the hemolymph acquires male factor activity. We suggest that male factor acts only indirectly on the salivary glands by somehow accelerating the rise in hemolymph ecdysteroid titer.

MATERIALS AND METHODS

Ticks

Adult *Amblyomma hebraeum* were obtained from a laboratory colony maintained in darkness at 26°C and >95% relative humidity. Unfed adult ticks (approximately 20–40 mg) were confined to the backs of rabbits as described earlier [10]. For rearing of mated females, equal numbers of males and females were confined together. For rearing of virgin females, the gonopores of the males were blocked with a drop of cyanoacrylate glue (Loctite Super Bonder 495, Loctite Corp., Newington, CT) before confining them with the females. Once all females had attached (12–24 h after placement) all males were removed. All females were later confirmed as virgin by the absence of spermatozoa in a seminal receptacle smear. Weights of females used in this study are as follows: females below the critical weight = 150–250 mg; females above the critical weight = 500–1,200 mg. The normal weight range for replete females of this species is about 1,500–3,000 mg.

Assay for Salivary Gland Secretory Competence

Fluid secretory competence was determined as follows: salivary glands were excised from ticks and the main duct ligated with one strand of an 8-0 surgical silk thread (Davis and Geck, Pearl River, NY) which was previously separated into 3 strands. Glands were then transferred to TCM 199 (Gibco Inc., Grand Island, NY) without sodium bicarbonate, buffered with 2.09 g/l

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*Abbreviations used: buffer:BSA = borate buffer:bovine serum albumin; 20-OHE = 20-hydroxyecdysone; LGT = low-gelling-temperature agarose; MOPS = 3-[N-morpholino]-propanesulphonic acid; pg EE/μl = pg 20-hydroxyecdysone equivalents/μl; TCM 199 = tissue culture medium 199; TSGDF = tick salivary gland degeneration factor.

MOPS (Sigma Chemical Co., St. Louis, MO) and osmotically adjusted to 360 mosmol/l with 2.1 g/l NaCl (pH adjusted to 7.3 with NaOH). Glands were gently blotted to remove excess medium and wet weights were measured to the nearest 0.01 mg. Glands were then incubated in constantly stirring TCM 199 containing 10 µM dopamine (Sigma). After 10 min, glands were removed, blotted, and reweighed. The wet weight increase of the gland was used as an indicator of secretory competence [1,5].

Injection of Male Reproductive Tracts

Males, fed for at least 5 days, were glued to a Petri dish and the reproductive tracts (testis/vas deferens/ejaculatory duct) dissected out in ice cold 1.2% NaCl (saline). The tissue was homogenized in ice cold saline (10 µl/gonad) and centrifuged at 8,000 g for 5 min. The supernatant (crude extract) was then injected into virgin females (2 gonad equivalents per female) using a 100 µl syringe equipped with a 30 gauge needle. Injection into the ticks was made through the camerostomal fold. Vehicle-injected controls received an equal volume of saline.

Collection of Tick Hemolymph

Ticks were washed with distilled water, glued to a Petri dish, and anaesthetized under ice for 15–20 min. Using a razor blade scalpel, an incision was made through the integument along each of the dorsolateral margins and gentle pressure was applied to the dorsal surface. The hemolymph exuding from these wounds was collected in 10 µl volumetric capillary tubes. For RIA of ecdysteroids, hemolymph was transferred directly into glass distilled methanol (BDH Inc., Toronto, Canada) and stored at − 12° C. For hemolymph injection experiments, 25 µl hemolymph (pooled when necessary) from donor ticks was immediately injected into each recipient virgin female using a 100 µl syringe equipped with a 30 gauge needle.

Ecdysteroid RIA

The RIA procedure used was that of Kaufman [11]. Ecdysteroids were extracted from hemolymph samples in 100% glass distilled methanol, evaporated under vacuum, and stored at − 12°C until assayed. At the time of assay, samples were reconstituted in an appropriate volume of buffer:BSA to allow at least three 75 µl replicates, yet maintain a predicted ecdysteroid content within the sensitivity of the RIA (10–500 pg ecdysteroid/replicate). To each replicate was added 90 µl radiolabelled ecdysone (45 Ci/mmol α-[23,24-^{3}H(N)]-ecdysone in buffer:BSA, approximately 8,000 cpm; NET 621, New England Nuclear, Boston, MA), and 45 µl antibody (final dilution 1:3,700 E-22 succinylthyroglobulin in buffer:BSA; antibody was the gift of L.I. Gilbert, University of North Carolina). After incubating for 4 h to overnight, we separated antibody-bound ligand from free ligand by adding 20 µl of 5% protein A (prepared from *Staphylococcus aureus* by Dr. J. Campbell, University of Alberta, according to the method of Kessler [12]), centrifuging at 8,000 g for 5 min, and aspirating away the supernatant. The pellet was resuspended in 50 µl distilled water, transferred to minivials containing 5 ml scintillation cocktail (Scintiverse E; Fisher Scientific, Nepean, Ontario, Canada) and radioactivity monitored on a scintillation counter. For each RIA, a standard curve was run concurrently, using 20-OHE as standard (Simes, Milan, Italy). Because the antibody shows cross-reactivity with many side chain modified ecdysteroids [13], all data are expressed as pg EE/μl.

Organ Culture of Salivary Glands

The organ culture technique was that of Kaufman [14]. Ticks were first prepared for culture by surface sterilization, 1 min each in 70% ethanol and 1% thimerosal, then transferred to a sterile laminar-flow culture hood. Ticks were glued to a sterile plastic Petri dish, covered in sterile TCM 199, and the salivary glands dissected out. Salivary glands were washed 3 times in TCM 199 (the third wash also containing the test concentration of 20-OHE), and transferred onto a prepared agar substrate. The agar substrate consisted of equal volumes of 2% (w/v) LGT (Miles Laboratories, Elkhart, IN), millipore filter sterilized (pore size 0.22 μm) double strength TCM 199, 100 μg/ml gentamicin (Sigma), and 2 × the desired 20-OHE concentration to be tested. This gave a final working substrate of single strength TCM 199 containing 1% LGT, 50 μg/ml gentamicin, and 1 × the 20-OHE concentration to be tested. Above this agar substrate, a volume of sterile TCM 199 (containing 50 μg/ml gentamicin and the desired 20-OHE concentration) was added to sufficiently cover the transplanted salivary glands (approximately 500 μl). Cultures were incubated at 26°C, >95% relative humidity for 4 days, at which time the salivary glands were tested for fluid secretory competence as described above.

Statistics

All data are reported as mean ± S.E. (n). Statistical significance was determined by one way analysis of variance (ANOVA) or Mann-Whitney U nonparametric test (using Statview™ SE + Graphics, Abacus Concepts Inc., CA) on a Macintosh computer. Statistical significance is indicated as follows: $^{*}0.05 > P > 0.01$; $^{**}0.01 > P > 0.001$; $^{***}P < 0.001$.

RESULTS

Male Factor Activity in the Hemolymph

Figure 1 indicates that male factor[1] appears in the hemolymph after copulation. Injection of virgin females with hemolymph from mated females, either above or below the critical weight (1.15 ± 0.20 and 0.56 ± 0.31 mg/gland/10 min, respectively) caused a significant decrease in fluid secretory competence compared to virgin females that received saline alone (2.80 ± 0.40; $P < 0.001$). This decreased fluid secretory ability, caused by the injection of hemolymph from mated donors, was not significantly different from saline injected mated controls. Injection of hemolymph from virgin donors above the critical weight

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[1] Throughout this paper, for simplicity, the term "male factor" refers either to male factor itself, or any other substance possessing male factor activity. It should be understood that we have not yet determined whether male factor activity of hemolymph or other tissues is due to the same substance found in the testicular fluid.

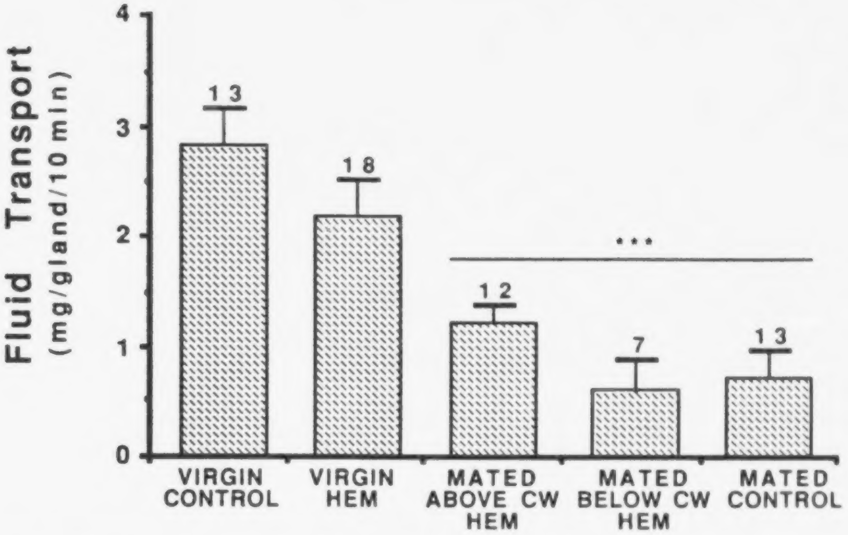

Fig. 1. Male factor activity of female hemolymph. Virgin females above the critical weight were injected with 25 μl hemolymph from donor females and their salivary glands assayed for secretory competence 4 days later. Donor females were: virgin females (VIRGIN HEM), mated females above the critical weight (MATED ABOVE CW HEM), or mated females below the critical weight (MATED BELOW CW HEM). VIRGIN CONTROL = virgin females; MATED CONTROL = mated females, each above the critical weight and injected with 25 μl saline. Mean ± S.E. (n). Statistical significance is indicated for this and subsequent figures as follows: *0.05 > P > 0.01; **0.01 > P > 0.001; ***P < 0.001.

did not cause a significant decrease in fluid secretory competence (2.16 ± 0.33 mg/gland/10 min).

Sensitivity of Salivary Glands to 20-OHE

With the hypothesis that male factor might act by sensitizing the salivary glands to ecdysteroids, we tested the efficacy of 20-OHE to induce salivary gland degeneration in mated or virgin glands after 4 days of organ culture. The dose response curves obtained for mated and virgin females were virtually identical (Fig. 2). Although the salivary glands from virgin females initially secreted at a higher rate than those of mated females (6.27 ± 0.28 vs. 4.90 ± 0.41 mg/gland/10 min; P < 0.001), thereafter, there was no significant difference between them. Exposure to as little as 30 pg 20-OHE/μl induced a significant decrease in fluid secretory ability (P < 0.001). Maximum response occurred at 100–500 pg 20-OHE/μl. As a further control, a series of nontreated mated females above the critical weight were assayed for fluid secretory competence and hemolymph ecdysteroid levels on day 4 postremoval. The salivary glands from these females secreted at a rate of 1.51 ± 0.14 mg/gland/10 min (n = 6) while exposed to final hemolymph ecdysteroid titers of 161 ± 37 pg EE/μl hemolymph (n = 3). These figures agree well with the data in Figure 2.

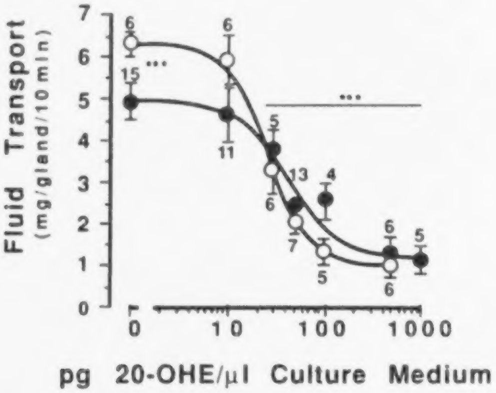

Fig. 2. Sensitivity of salivary glands to 20-OHE. Fluid secretory competence as a function of 20-OHE concentration in the culture medium following 4 days incubation. Open circles = glands from virgin females above the critical weight; filled circles = glands from mated females above the critical weight. Mean ± S.E. (n). Glands exposed to ≥ 30 pg 20-OHE/µl secreted significantly less than controls. Virgin controls (0 pg) secreted significantly more than mated controls. Statistical significance as in Figure 1.

Concentration Profile of Hemolymph Ecdysteroids

We next measured hemolymph ecdysteroid content of mated and virgin females up to 8 days postremoval to see if there were differences that might account for the delay in salivary gland degeneration in virgins. The ecdysteroid titer of hemolymph from virgin and mated females below the critical

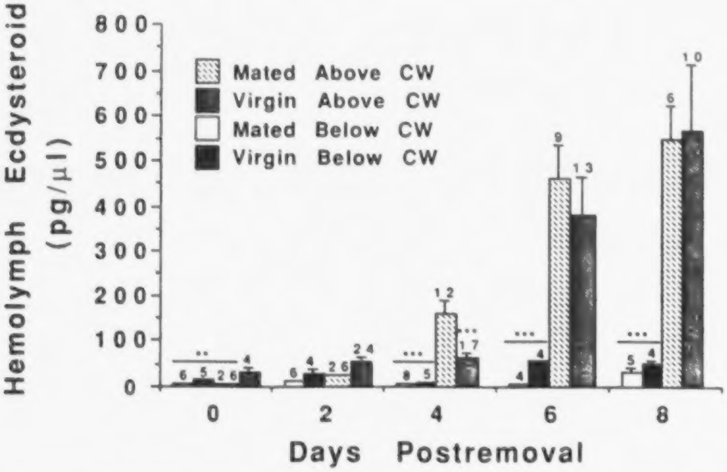

Fig. 3. Hemolymph ecdysteroid titer measured by RIA as a function of days postremoval from the host, for mated and virgin females above and below the critical weight. Mean ± S.E. (n). Statistical significances are as in Figure 1, and compared to the highest bar of each day post removal.

weight remained low throughout the 8 day period (< 50 pg EE/μl; Fig. 3). The rise in hemolymph ecdysteroid in virgin females above the critical weight was, however, significantly delayed compared to their mated counterparts (Fig. 3). Hemolymph ecdysteroid titers of mated females above the critical weight were initially low (10 ± 1 pg EE/μl); however, by day 2 they began to increase rapidly and by day 4 postremoval, reached levels of 162 ± 27 pg EE/μl. Ecdysteroid titers continued to increase, reaching levels of 550 ± 72 pg EE/μl by day 8. Conversely, ecdysteroid titers of virgin females on day 0 were significantly higher than that of mated females (30 ± 7 pg EE/μl; $P < 0.001$). The subsequent rise in ecdysteroid titer was, however, attenuated and did not significantly increase by day 4 ($P = 0.12$); the level was significantly lower than that of their day 4 mated counterparts (62 ± 9 vs. 162 ± 27 pg EE/μl, respectively; $P < 0.001$). Hemolymph ecdysteroid titers began to increase substantially only after day 4, and by day 8 postremoval, the hemolymph titer (567 ± 146 pg EE/μl) was not significantly different from that of their mated counterparts.

Effect of Male Factor on Hemolymph Ecdysteroid Titer

We next tested whether injections of male factor into virgins above the critical weight would hasten the rise of hemolymph ecdysteroid titer. Injection of 2 male reproductive tract equivalents into virgin females above the critical weight caused a significant increase in hemolymph ecdysteroid titers by day 4 as compared to saline injected controls (130 ± 17 vs. 29 ± 7 pg EE/μl, respectively; $P < 0.001$; Fig. 4). This elevated hemolymph ecdysteroid level was not significantly different from day 4 saline injected mated controls (168

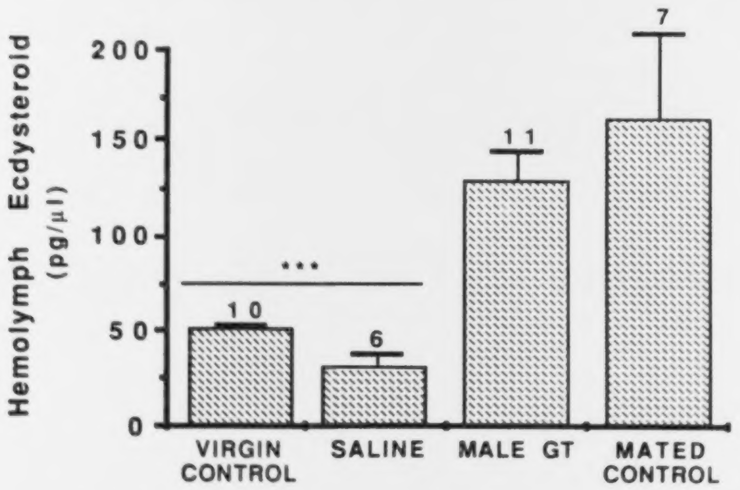

Fig. 4. Effect of male factor on hemolymph ecdysteroid titer. Hemolymph ecdysteroid titer was measured 4 days after removal from the host in virgin females above the critical weight, after the injection of either a male reproductive tract homogenate (MALE GT), or saline (SALINE). VIRGIN CONTROL = virgin females without injection; MATED CONTROL = saline injected mated females. Mean ± S.E. (n). Statistical significances are as in Figure 1.

± 39 pg EE/μl hemolymph). We measured the ecdysteroid content of the injected homogenates to determine if that might account for this significant rise in hemolymph ecdysteroid content. RIA indicated an ecdysteroid content of only 9 ± 6 pg EE/homogenate injected (n = 3). Considering that the hemolymph volume represents about 23% body weight of partially fed ticks and about 10% body weight of engorged ticks [15], the 9 pg of injected ecdysteroid would have increased the hemolymph titer of a 1,000 mg tick by less than 0.1 pg EE/μl.

DISCUSSION

During copulation, the male deposits a spermatophore onto the ventral surface of the female, which is then internalized and stored in the seminal receptacle [16]. Male factor is probably a component of the testicular fluid [9]. It has been suggested that male factor leaves the seminal receptacle, enters the hemolymph, and reaches its target tissue(s) as a humoral factor. Harris and Kaufman [5] ruled out the possibility of male factor acting by a neural mechanism, since severing all nerves from the synganglion to the seminal receptacle did not delay salivary gland degeneration. A humoral mechanism was indicated, since surgical removal of the seminal receptacle in mated females and replacement with a male genital tract homogenate [5], or injection of a male reproductive tract homogenate directly into the hemocoel of virgin females [9] mimicked the effect of mating. Here we show that injection of hemolymph from mated donors directly into the haemocoel of virgin females above the critical weight is also sufficient to initiate salivary gland degeneration (Fig. 1).

We emphasize here that male factor is distinct from TSGDF for at least three reasons: (1) male factor is abundant in the hemolymph of mated females below the critical weight (Fig. 1), and salivary gland degeneration does not occur in such females for at least 14 days [5]; (2) evidence from in vivo and in vitro studies indicates that TSGDF is an ecdysteroid [4,6,17]. Although the male reproductive tract does contain a small amount of ecdysteroid (see above), this exogenous source of ecdysteroid is insufficient to account for the observed salivary gland degeneration; and (3) heat lability and sensitivity to proteinase K digestion indicate that male factor is a protein [9].

Evidence presented here indicates that male factor does not act by sensitizing the salivary glands to the action of ecdysteroids (e.g., by up-regulation of ecdysone receptors). Thus, salivary glands of virgin females are equally sensitive to 20-OHE as those of mated females (Fig. 2). Instead, male factor appears to act indirectly on the salivary glands by hastening the appearance of ecdysteroids in the hemolymph (Fig. 4). The increase in hemolymph ecdysteroid titer in virgins caused by male factor is sufficient to account for the loss of salivary gland secretory competence in virgin females by day 4. Our organ culture evidence suggests that 30 pg 20-OHE/μl culture medium is sufficient to initiate a reduction in fluid secretory competence, while a complete loss occurs at 100–500 pg 20-OHE/μl. These figures coincide well with the original report by Harris and Kaufman [4]. In vivo experiments by Kaufman [17] correlated natural hemolymph ecdysteroid titers directly with salivary gland secretory competence. He demonstrated a partial reduction in salivary gland function at a mean hemolymph ecdysteroid titer of about 90 pg EE/µl, while degeneration was almost complete at an ecdysteroid titer of 140 pg EE/µl hemolymph; these values are well in agreement with our mated controls of Figure 2 (161 pg EE/µl). Finally, virgin females above the critical weight show no salivary gland degeneration by day 4; a significant degree of degeneration is apparent only by day 6 [9]. During this time, the hemolymph ecdysteroid titer rises from 60 pg EE/µl to 379 pg EE/µl (Fig. 3). Considering the fairly large variability inherent in RIA measurements, and that the antibody we used shows cross-reactivity with many side chain modified ecdysteroids [13], we feel that the in vitro and in vivo data are in general agreement. Taking all the foregoing into consideration, the induced rise in hemolymph ecdysteroid titer by male factor injected into large virgins (Fig. 4) does seem sufficient to account for the observed salivary gland degeneration and thus, the proposed mechanism of action. We still do not know how male factor modulates hemolymph ecdysteroid titer. Until an ecdysteroid synthesizing organ is positively identified in adult ticks, the nature of male factor's direct action will remain elusive.

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